**University of Bremen**

**Center for Human Genetics**

# Quantitative analysis of *thyroid adenoma associated* (*THADA*) and *high-mobility group AT-hook 2* (*HMGA2*) in dedifferentiated and extra-embryonic human tissues

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Bremen, den 27.04.2015

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(Lars-Gerrit Kloth)

# Contents

## Abbreviations

| | |
|---|---|
| °C | Degree Celsius |
| β-HCG | Human choriogonadotropin subunit beta |
| µl | Microliter |
| µm | Micrometer |
| 3'-UTR | Three prime untranslated region |
| A | Adenine |
| aa | Amino acid |
| AD | After delivery |
| AFP | Alpha-fetoprotein |
| AIX | Ampicillin, isopropyl β-D-1-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) |
| Ala | Alanine |
| ALL | Acute lymphocytic leukemia |
| ALT | Atypical lipomatous tumors |
| AML | Acute myeloid leukemia |
| ARM repeat | Armadillo repeat |
| ATC | Anaplastic thyroid carcinoma |
| ATF-2 | Activating transcription factor 2 |
| bp | Base pair |
| cAMP | Cyclic adenosine monophosphate |
| CC | Choriocarcinoma |
| CCNB2 | Cyclin B2 |
| CD117 | V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) |
| CD30 | Tumor necrosis factor receptor superfamily, member 8 (TNFRSF8) |
| cDNA | Complementary deoxyribonucleic acid |
| CGA | Calendar gestational age |
| CHM | Complete hydatidiform mole |
| CIAP | Calf-intestinal alkaline phosphatase |
| CIS | Carcinoma in situ |
| c-KIT | V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) |
| CLL | Chronic lymphocytic leukemia |

| | |
|---|---|
| CML | Chronic myeloid leukemia |
| CREB | Cyclic adenosine monophosphate (cAMP) response element |
| $C_T$ | Cycle threshold |
| $ddH_2O$ | Double-distilled water |
| DNA | Deoxyribonucleic acid |
| DR5 | Death Receptor 5 |
| E2F1 | E2F transcription factor 1 |
| EC | Embryonal carcinoma |
| E-cadherin | Epithelial cadherin |
| EGF | Epidermal growth factor |
| EMT | Epithelial to mesenchymal transition |
| ERCC1 | Excision repair cross-complementation group 1 |
| EST | Expressed sequence tag |
| EVT | Extravillous trophoblast |
| FFPE | Formalin-fixed paraffin-embedded |
| FGF4 | Fibroblast growth factor 4 |
| FISH | Fluorescence in situ hybridization |
| FNA | Fine-needle aspiration |
| FOXC1 | Forkhead box C1 |
| FTC | Follicular thyroid carcinoma |
| G | Guanine |
| GCNF | Germ cell nuclear factor (nuclear receptor subfamily 6, group A, member 1, NR6A1) |
| GFP | Green fluorescent protein |
| GTT | Gestational trophoblastic tumor |
| GWAS | Genome-wide association study |
| h | Hour |
| $H_2O_2$ | Hydrogen peroxide |
| HA | Hydropic abortion |
| HHEX | Hematopoietically expressed homeobox |
| Hlf | Hepatic leukemia factor |
| HMG | High mobility group |

## Abbreviations

| | |
|---|---|
| HMGA | High mobility group AT-hook |
| HMGA1 | High mobility group AT-hook 1 |
| HMGA1a | High mobility group AT-hook 1 isoform a |
| HMGA1b | High mobility group AT-hook 1 isoform b |
| HMGA1c | High mobility group AT-hook 1 isoform c |
| HMGA2 | High mobility group AT-hook 2 |
| HMGB | High mobility group box |
| HMGN | High mobility group nucleosomal binding domain |
| HPRT | Hypoxanthine phosphoribosyltransferase 1 (HPRT1) |
| hUCBSC | Human umbilical cord blood-derived stromal cell |
| IA | Induced abortion |
| ICD-O | International Classification of Diseases for Oncology |
| IGF2BP3 | Insulin-like growth factor 2 mRNA-binding protein 3 |
| IMT | Inflammatory myofibroblastic tumor |
| IMUP-2 | Immortalization-upregulated protein (chromosome 19 open reading frame 33, C19orf33) |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| ITGCNU | Intratubular germ cell neoplasia undifferentiated |
| kbp | Kilo base pairs |
| kDa | Kilo dalton |
| LB | Lysogeny broth |
| LD | Linkage disequilibrium |
| let-7 | Lethal-7 |
| LIN28 | Lin-28 homolog A |
| LPP | LIM domain containing preferred translocation partner in lipoma |
| M | Molar |
| MAPK | Mitogen-activated protein kinase |
| MDS | Myelodysplastic syndrome |
| min | Minute |
| miRNA | microRNA |
| ml | Milliliter |
| mRNA | Messenger RNA |

| | |
|---|---|
| MS | Multiple sclerosis |
| MTC | Medullary thyroid carcinoma |
| n | Number of samples |
| n.a. | Not available |
| NANOG | Nanog homeobox |
| NCBI | National Center for Biotechnology Information |
| Nek2 | NIMA-related kinase 2 |
| ng | Nanogram |
| NGS | Next-generation sequencing |
| NIS | Sodium-iodide symporter (solute carrier family 5, member 5, SLC5A5) |
| Nkx2-2 | NK2 homeobox 2 |
| Nkx2-5 | NK2 homeobox 5 |
| Nkx6-1 | NK6 homeobox 1 |
| nM | Nanomolar |
| NSCL/P | Nonsyndromic cleft lip with or without cleft palate |
| OCT3/4 | Octamer-binding transcription factor 4 (POU domain class 5, transcription factor 1, POU5F1) |
| ORF | Open reading frame |
| $p57^{KIP2}$ | Cyclin-dependent kinase inhibitor 1C (CDKN1C) |
| PAX8 | Paired box 8 |
| PCOS | Polycystic ovary syndrome |
| PCR | Polymerase chain reaction |
| PHM | Partial hydatidiform mole |
| PLAP | Placental alkaline phosphatase |
| PPARγ | Peroxisome proliferator-activated receptor gamma |
| PTC | Papillary thyroid carcinoma |
| qRT-PCR | Quantitative real-time polymerase chain reaction |
| $R^2$ | Coefficient of determination |
| RAD51L1 | RAD51 homolog B |
| RNA | Ribonucleic acid |
| ROC | Receiver-Operator-Characteristics |
| RQ | Relative quantification |

## Abbreviations

| | |
|---|---|
| rRNA | Ribosomal ribonucleic acid |
| RT-PCR | Reverse transcription polymerase chain reaction |
| s | Second |
| SA | Spontaneous abortion |
| SALL4 | Spalt-like transcription factor 4 |
| SNAIL1 | Snail family zinc finger 1 |
| SNP | Single nucleotide polymorphism |
| SOX2 | SRY (sex determining region Y)-box 2 |
| T2D | Type 2 diabetes |
| TAE | Tris base, acetic acid, ethylenediaminetetraacetic acid (EDTA) |
| TGCT | Testicular germ-cell tumor |
| TGF-ß | Transforming growth factor-beta |
| TGF-ßRII | Transforming growth factor-beta type II receptor |
| TGF-b1 | Transforming growth factor beta 1 |
| THADA | Thyroid adenoma associated |
| THADA-A1 | Thyroid adenoma associated variant |
| THADA-A2 | Thyroid adenoma associated variant |
| THADA-A3 | Thyroid adenoma associated variant |
| THADA-A3-Fus3p | Thyroid adenoma associated fusion transcript |
| THADA-Fus3p | Thyroid adenoma associated fusion transcript |
| THADA-Fus7p | Thyroid adenoma associated fusion transcript |
| Thr | Threonine |
| TIN | Testicular intraepithelial neoplasia |
| TPM | Transcripts per million |
| TSH | Thyroid-stimulating hormone |
| TTF1 | Transcription termination factor, RNA polymerase I |
| TTF-2 | Thyroid transcription factor 2 (Forkhead box E1, FOXE1) |
| U | Enzyme unit |
| w/ | with |
| X-Gal | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |
| YST | Yolk sac tumor |

# 1. Introduction

Undisclosing the sequence of the human genome was finished in principle in 2003 (Schmutz *et al*., 2004). Since then, the challenge for science is not only elucidating the position and structure of the genes in the succession of the base pairs, but rather understanding the role of each of these genes in the complex interplay in the cellular network of the human body. In this thesis two genes, *thyroid adenoma associated* (*THADA*) and *high-mobility group A2* (*HMGA2*), were analyzed mainly in context with their expression in dedifferentiated and extra-embryonic human tissues.

*THADA* (*thyroid adenoma associated*), located on the short arm of chromosome 2, was discovered by Rippe *et al*. (2003) as the target gene involved in translocations commonly found in thyroid adenomas. Besides polysomy 7 and 19q13 aberrations, 2p21 rearrangements were identified as the third most frequent mutation in hyperplasias and benign follicular tumors of the thyroid (Bondeson *et al*., 1989; Teyssier *et al*., 1990; Dal Cin *et al*., 1992; Belge *et al.*, 1994; Belge *et al*., 1998; Bol *et al*., 1999). Identification of *THADA* became possible after the breakpoint was narrowed down to a region of 450 kbp (Bol *et al*., 2001).

Except for one entry in the GenBank database by Puduvalli and Ridgway (GenBank accession reference note), describing an interaction of THADA with death receptor DR5 in a two-hybrid experiment, no information about its function was available prior to this work.

In conjunction with the studies presented in this thesis, research by Drieschner *et al*. (2007), analyzing the structure of *THADA* in several vertebrates (*Canis familiaris*, *Chlorocebus aethiops*, *Gallus gallus*, and *Mus musculus*), revealed similarities to ARM repeat structures. Also identified was the most conserved part of the protein (aa 1033 - 1415 *Homo sapiens*) hinting at a putative important functional domain. 2p21 translocations, resulting in truncations of *THADA*, disrupt this domain. This might cause a loss of function contributing to the development of the above mentioned follicular thyroid neoplasias (Drieschner *et al*., 2007).

Drieschner *et al.* (2006) also reported a follicular thyroid adenoma with a translocation t(2;20;3)(p21;q11.2;p25), which showed an intronic sequence of *PPARγ* fused to exon 28 of *THADA*. The authors concluded that the close surrounding of *PPARγ* is likely to be a

breakpoint hot spot region, which leads to recurrent alterations of this gene in follicular thyroid neoplasias with or without the involvement of *PAX8* (Drieschner *et al*., 2006).

Nikiforov *et al.* (2014) used next-generation sequencing (NGS) on 143 fine-needle aspirations (FNA) of thyroid nodules. By applying a panel with simultaneous testing for point mutations in 13 genes and for 42 types of gene fusions that occur in thyroid cancer, in five cases a fusion gene involving *THADA* and *IGF2BP3* as the unique diagnostic event was detected (Nikiforov *et al.,* 2014). The authors concluded that comprehensive genotyping of thyroid nodules using a broad NGS panel should facilitate the optimal management of patients with indeterminate diagnosis after an FNA.

In a study investigating a t(2;11)(p21;q23) mutation in 19 myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) cases (Bousquet *et al*., 2008), *THADA* expression was measured due to its location close to the breakpoint. No difference in expression level could be detected for the *THADA* mRNA, the authors deemed the upregulation of miR-125b-1 to represent a new mechanism of myeloid cell transformation. An involvement of *THADA* in a translocation t(2;3)(p15-21;q26-27) in two AML patients was detected by Trubia *et al*. (2006). Since the orientation of *THADA* was opposite to the one of its fusion partner, a contribution to a fusion protein was deemed unlikely by the authors (Trubia *et al*., 2006).

Additionally, *THADA* has been associated with several diseases, namely type 2 diabetes (T2D) (Zeggini *et al*., 2008), polycystic ovary syndrome (PCOS) (Chen *et al*., 2011), and nonsyndromic cleft lip (Ludwig *et al*., 2012). Zeggini *et al.* carried out a meta analysis of three genome-wide association studies (GWAS) investigating about 2.2 million single nucleotide polymorphisms (SNP) in more than 10,000 individuals of European descent for a possible association with T2D. A *THADA* allele characterized by a SNP in exon 24 turned out to be significantly associated with an elevated risk of developing T2D (Zeggini *et al*., 2008). These initial findings were corroborated by several other investigations (Parikh *et al*., 2009; Simonis-Bik *et al*., 2010; Voight *et al*., 2010; Gupta *et al*., 2012; Nitert *et al*., 2012; Franceschini *et al*., 2013; Ho *et al*., 2013; Pendse *et al*., 2013). The same association found in a European cohort as described in Zeggini *et al*. (2008) was detected in Indian sib pairs by Gupta *et al*. (2012) and in American Indians by Franceschini *et al*. (2013). An association between *THADA* risk allele and ß-cell mass (Simonis-Bik *et al*., 2010), and function (Voight *et al*., 2010) was reported. Other investigations found

differences in methylation status (Nitert *et al*., 2012) and expression level of *THADA* in ß cells in pancreatic islets in humans (Parikh *et al*., 2009) and adipose tissue and pancreatic islets in mice (Ho *et al*., 2013) in relation to T2D. Nevertheless, in a substantial amount of studies no correlation of *THADA* with T2D could be detected (Grarup *et al*., 2008; Staiger *et al*., 2008; Boesgaard *et al*., 2009; Sanghera *et al*., 2009; Stančáková *et al*., 2009; Vangipurapu *et al*., 2011; Almawi *et al*., 2013). No correlation was found for the *THADA* risk variant for obesity, insulin secretion and insulin sensitivity in a Danish (Grarup *et al*., 2008), and a German (Staiger *et al*., 2008) cohort. The same negative results for the insulin related T2D traits were observed in studies testing Finnish men (Stančáková *et al*., 2009), European individuals (Boesgaard *et al*., 2009) and Asian Indian Sikhs (Sanghera *et al*., 2009). In a case-control association study with Lebanese-Arabs no association between *THADA* risk allele and T2D was found (Almawi *et al*., 2013). In several cases authors suggest that their negative findings may be explained by a low statistical power, i.e. an insufficient study size relative to the allele frequency (Grarup *et al*., 2008; Sanghera *et al*., 2009; Stančáková *et al*., 2009; Almawi *et al*., 2013). In one case, because of an extremely low minor allele frequency, *THADA* was excluded from a study (Kang *et al*., 2009). Additionally, two studies tested if *THADA* can have an indirect influence on diabetes (Zhao *et al*., 2010; Hotta *et al*., 2012), but the former study did not reveal a correlation between higher pediatric body mass index, a known risk factor in the development of T2D in later life, and a *THADA* risk variant. Hotta *et al*. genotyped Japanese individuals to examine T2D susceptibility loci, including *THADA*, and visceral fat accumulation, which has an important role in the development of several metabolic disorders, like T2D, but found none. Also, no association between *THADA* and type 1 diabetes was reported by Raj *et al*. (2009).

Patients with diabetes mellitus carry an increased risk of developing colon and rectal cancer (Larsson *et al*., 2005). In a case-control study by Cheng *et al*. (2011) testing known risk alleles of T2D, *THADA* rs7578597 Thr1187Ala missense polymorphism had the strongest association for colon cancer, but the low risk allele for T2D turned out to be the high risk variant for colon cancer. Congruously, the authors suggested a different mechanism for each disease, driven by different biological effects of THADA and its variants, respectively.

PCOS is a very common chronic endocrine disorder, affecting up to 20 % of females in

reproductive age (Teede *et al*., 2010). An association between PCOS and three SNP-loci in *THADA* ORF was first reported by Chen *et al*. (2011), investigating Han Chinese individuals. These results were corroborated in a family-based analysis on the same ethnic group (Zhao *et al*., 2012). This association was also found in a European derived (Goodarzi *et al*., 2012), and a Dutch (Louwers *et al*., 2013) cohort. Despite the confirmed relation between genotype and PCOS, Goodarzi *et al*. could not find a correlation between SNPs and PCOS traits like testosterone levels. Contradictory to these results, Cui *et al*. (2013) reported a correlation between SNPs located in *THADA* and increased testosterone, among other traits. Other studies failed to find any correlation (Lerchbaum *et al*., 2011; Eriksen *et al*., 2012; Wang *et al*., 2012; Welt *et al*., 2012). Lerchbaum *et al*. tested rs13429458, one of the risk alleles of *THADA* reported by Chen *et al*. (2011), in a Caucasian cohort; Eriksen *et al*. (2012) investigated the same variant in Danish descendants with PCOS or hirsutism. In both cases the genotype distribution and allele frequency as well as the endocrine or metabolic parameters did not differ significantly between patient and control groups. Wang *et al*. (2012) conducted a case-control study on Han Chinese women with endometrial carcinomas but found no statistical difference for rs13429458. A study testing all three SNPs located in *THADA* on women of European ancestry performed by Welt *et al*. (2012) revealed no association with PCOS, but for one of the alleles testosterone levels were higher. The authors suggested a possible ethnic specificity (Welt *et al*., 2012). Contrary to this, Brower *et al*. (2014) concluded that *THADA* loci are likely to play important roles in PCOS across populations.

PCOS can lead to insulin resistance and T2D (Wild *et al*., 2000; Carmina *et al*., 2009; Teede *et al*., 2010). A study conducted on women of European ancestry tested for the possibly T2D associated *THADA* SNP rs7578597 in correlation to PCOS, but no association could be detected (Saxena, Welt, 2013). The authors also reported no linkage disequilibrium (LD) between the possible T2D alleles with the PCOS variants (Saxena, Welt, 2013), corroborating the results reported by Goodarzi *et al*. (2012), and partly those of Chen *et al*. (2011), who in addition found such an LD in a cohort consisting of residents of Utah, USA, with Northern and Western European ancestries, opposite to those of a Han Chinese cohort.

*THADA* in correlation with nonsyndromic cleft lip with or without cleft palate (NSCL/P) was first mentioned in a GWAS testing individuals of central European origin by Mangold *et al*.

(2010). But the association of rs7590268, located in intron 31 of *THADA*, and NSCL/P turned out to be not significant (Mangold *et al*., 2010). Later, this association was reported as being significant by Ludwig *et al*. (2012), who conducted a meta-analysis of the GWAS mentioned in Mangold *et al*. and one presented in Beaty *et al*. (2010), which consisted of case-parent trios of European and Asian ancestry. While rs7590268 achieved no significance in a replication study by Beaty *et al*. (2013), several nearby SNPs did. A case-control study of a Chinese Han cohort by Pan *et al*. (2013) confirmed the association of *THADA* and NSCL/P and elucidated an association between rs7590268 and a positive family history of NSCL/P.

*THADA* was also mentioned in correlation with multiple sclerosis (MS) (Patsopoulos *et al*., 2011). In a meta-analysis of GWA studies searching for SNPs associated with MS, rs6718520, located about 132 kbp away from *THADA*, was one of three newly discovered SNPs.

A *THADA* variant has also been associated with elevated risk of cancer. In a large scale GWAS with each more than 20,000 patients and controls, Eeles *et al*. (2009) found *THADA* SNP rs1465618 to be highly significantly associated with prostate cancer. This correlation was later verified by Lindstrom *et al*. (2011), even though no association between this variant of *THADA* and Gleason score and stage of the disease could be detected. No correlation at all for rs1465618 was found in a study restricted to African American men (Haiman *et al*., 2011). In a study solely investigating this SNP, Zhao *et al*. (2014) found a significant association with prostate cancer risk and aggressiveness in a Chinese population.

While the majority of studies investigated *THADA* in humans, Soller *et al*. (2008) reported the successful mapping of *THADA* in the canine genome to chromosome 10p25. The authors argued that this region is not a hotspot for mutations in thyroid adenomas of the dog.

In opposite to *THADA*, a significantly larger amount of research had already been undertaken on *high-mobility group A2* (*HMGA2)*. Human HMGA2 was first detected by Giancotti *et al*. (1991) in a hepatoma cell line. It belongs to a protein family of three main subgroups. All are characterized by a small size of around 100 to 200 aa (Thomas, 2001; Cleynen, Van de Ven, 2008; Pogna *et al*., 2010), and a high percentage of charged amino

residues (Cleynen, Van de Ven, 2008). HMG proteins are highly abundant non-histone chromosomal proteins (Pogna *et al*., 2010). Members of the HMGN subfamily contain a nucleosome binding domain (Rochman *et al*., 2010), HMGB proteins are characterized by a HMG-box motif (Thomas, 2001). The HMGA subgroup consists of four proteins, HMGA1a, HMGA1b, HMGA1c (deriving from alternative splicing of one transcript), and HMGA2, these are encoded by two genes. All HMGA proteins contain a functional sequence motif, the AT-hook, which enables them to bind to specific DNA or chromatin structures (Cleynen, Van de Ven, 2008). Three AT-hooks are separately encoded in the first three exons, exon IV contains the sequence for a spacer region of eleven amino acids missing in *HMGA1* (Chau *et al*., 1995). For all *HMGA* members, exon V encodes the acidic tail (Chau *et al*., 1995). Due to diverging donor and acceptor splice sites and a different stop codon, the 3'-part of HMGA1c differs significantly from the other HMGA-proteins (Cleynen, Van de Ven, 2008). The acidic part of the protein is thought to be responsible for modulation of interactions with other proteins (Noro *et al*., 2003). This reciprocation can lead to the formation of complex molecular aggregates called transcription complexes (Cleynen, Van de Ven, 2008). Protein-protein-interactions involve HMGA in several molecular networks (reviewed in Sgarra *et al*., 2010). HMGA proteins have only very little secondary structure when in solution but if bound to DNA or other proteins a transition to ordered structure takes place (reviewed in Reeves, 2001). They belong to the most highly adducted proteins of the nucleus, posttranscriptional modifications can severely impact their affinity to bind to other proteins and DNA (Cleynen, Van de Ven, 2008; Zhang, Wang, 2008).

Studies on knock-out mice showed that *HMGA2* plays a crucial role in early development, a null mutant genotype resulted in the pygmy phenotype (Zhou *et al*., 1995). Hirning-Folz *et al*. (1998) found high expression throughout murine embryos at an early developmental stage with a correlation between the expression of *HMGA2* and high proliferative tissue activity.

*HMGA2* is downregulated in adult tissues (Rogalla *et al*., 1996), with the exception of spermatocytes and spermatides in testis (Kloth, Gottlieb, *et al*., 2015). Reexpression, truncation or generation of fusion transcripts through rearrangements is frequently found in several benign tumors, like lipomas and uterine leiomyomas (reviewed in Fedele, Fusco, 2010). Located in the chromosomal region 12q14-15 in humans, *HMGA2* is frequently

involved in translocations in benign neoplasias. In these cases the mutations often occur in the very large third intron (Schoenmakers *et al*., 1995; Mine *et al*., 2001). This causes a separation of the AT-hooks from the acidic tail resulting in a fusion protein with ectopic sequences added to the DNA-binding section of HMGA2. In lipomas, a fusion with part of LPP (LIM domain containing preferred translocation partner in lipoma) has been reported (Petit *et al*., 1996). In this case the two most carboxyterminal LIM-domains from LPP are fused to the three AT-hooks from HMGA2, thus creating an aberrant transcription factor likely contributing to lipomagenesis (Crombez *et al*., 2005). In uterine leiomyomas, a different pathological mechanism was proposed as translocation breakpoints were detected outside of *HMGA2*, indicating a dysregulation of *HMGA2* without a fusion transcript (Schoenberg Fejzo *et al*., 1996). In concordance with these results, Quade *et al*. (2003) deemed a fusion of *HMGA2* with the preferential translocation partner *RAD51L1* unnecessary for the development of this type of benign tumor. In the majority of other cases involving a fusion gene, only a few in-frame amino acids or out-of frame sequences are added to the truncated *HMGA2* (Cleynen, Van de Ven, 2008), suggesting another mechanism contributing to or initializing tumor development.

*HMGA2* turned out to be a major target for let-7 miRNA (Boyerinas *et al*., 2008), multiple target sites in the 3'-UTR of *HMGA2* mRNA were found (Lee, Dutta, 2007). Mayr *et al*. (2007) proposed that by repressing *HMGA2*, let-7 acts as a tumor suppressor gene and that in various human tumors the loss of let-7 repression is a major mechanism of oncogenic *HMGA2* translocations. Rearrangements of *HMGA2* are primarily but not exclusively found in benign tumors. Tallini *et al*. (1997) detected the activation of *HMGA2* through cytogenetic amplifications in malignant atypical lipomatous tumors (ALT), Kazmierczak *et al*. (1999) reported a case of an inflammatory myofibroblastic tumor (IMT) with an intragenic rearrangement. High expression of *HMGA* is mainly found in malignant tumors (reviewed in Cleynen, Van de Ven, 2008; Fedele, Fusco, 2010). This overexpression can induce oncogenic transformation of the affected cell through several mechanisms (Fedele, Fusco, 2010). By upregulation of proteins involved in the control of the cell cycle like E2F1, cyclinA, and CCNB2, *HMGA2* overexpression can lead to the activation of the cell cycle (reviewed in Fedele, Fusco, 2010). HMGA proteins can also influence the transformation through inhibition of DNA repair. Borrmann *et al*. (2003) elucidated the downregulation of ERCC1, a protein involved in the nucleotide excision

repair, by high expression of *HMGA2*. For *HMGA1*, several target genes participating in double-strand break repair, homologous recombination, and base excision repair have been reported (reviewed in Reeves, Adair, 2005). Epithelial to mesenchymal transition (EMT) is a key event in embryogenesis, because in its absence development stops at the gastrula stage due to lack of transformation of epithelial cells to the motile and unpolarized mesenchymal counterparts (reviewed in Thiery, Sleeman, 2006). EMT takes place in numerous stages during developmental morphogenesis (reviewed in Nakaya, Sheng, 2013). This is also the case for extra-embryonic tissues. The change of the villous cytotrophoblast from a coherently layered alignment to an extravillous state through EMT to enable a fetalomaternal exchange is of critical importance for a successful pregnancy (Vićovac, Aplin, 1996; Kokkinos *et al*., 2010). In tumorigenesis this latent developmental program becomes reactivated, multiple signaling pathways can contribute to EMT (reviewed in Huber *et al*., 2005). Thuault *et al*. (2006; 2008) elucidated that HMGA2 is necessary and sufficient for transforming growth factor-beta (TGF-ß)-induced EMT. The authors outlined an EMT inducing pathway in which *HMGA2* gets indirectly activated by TGF-ß and consequently, through gene-specific regulation of the expression of SNAIL1, leads to severe E-cadherin suppression and the EMT phenotype (Thuault *et al*., 2008). *HMGA2* has been suggested as a therapeutic target in pancreatic cancer, a highly malignant neoplasia due to elevated mitotic activity and EMT (Watanabe *et al*., 2009). At the invasive front of a tumor, a one-cell-thick layer of dedifferentiated and incoherent cells can be found (Morishita *et al*., 2013). In several tumor types *HMGA2* expression was detected at this layer (Morishita *et al*., 2013). The authors reported the activation of the expression of TGF-ßRII (TGF-ß type II receptor), preferably where the tumor cells exhibit the EMT. Recently, *HMGA2* expression has also been associated with EMT in bladder (Ding *et al*., 2014), gastric (Zha *et al*., 2013), liver (Luo *et al*., 2013), and prostate cancer (Zhu *et al*., 2013).

As mentioned above, *HMGA2* is known to play an important role in the early development of an individual. Part of this thesis focused on the detection of *HMGA2* in the placenta. Besides studying normal gestations, another investigation dealt with a certain type of pathological pregnancies. In relatively rare cases of pregnancy, i.e. a molar pregnancy, the embryo is lacking. A molar pregnancy is a relatively uncommon occurrence, in North America and Europe 0.5-1.95 incidents per 1,000 pregnancies have been reported, in

Japan 2.0 to 2.5 per 1,000 (Bracken, 1987; Jeffers *et al*., 1993; Palmer, 1994). Hydatidiform moles are classified as partial or complete moles. The closely related diploid hydropic abortion is not considered molar. Complete hydatidiform moles (CHM) consist of only the trophoblast and are usually diploid, but contain only paternal DNA. Partial hydatidiform moles (PHM) also contain the anlage of the embryo and are in most cases triploid with chromosomes deriving from both parents. In 10 to 20 % of the CHMs a gestational trophoblastic neoplasm, i.e. a malignant tumor, develops (Berkowitz, Goldstein, 1996; van de Kaa *et al*., 1996; Sebire, Seckl, 2008; Berkowitz, Goldstein, 2009; Kaneki *et al*., 2010). The risk is considerably lower (Bagshawe *et al*., 1990; Cheung *et al*., 2004; Feltmate *et al*., 2006; Hancock *et al*., 2006), or possibly nonexistent (Jeffers *et al*., 1993; van de Kaa *et al*., 1996; Niemann *et al*., 2007; Kaneki *et al*., 2010; reviewed in Hoffner, Surti, 2012) in case of the PHM. Hydropic abortions bear no risk of a malignant degeneration (Chen *et al*., 2012). Even though gestational trophoblastic tumors (GTT) are usually highly susceptible to chemotherapy, 12 % of the women with a high risk form of a GTT die after developing this type of cancer (Bower *et al*., 1997). In addition, choriocarcinoma bears an elevated risk for the recipient after transplantation of an organ (Marsh *et al*., 1987; Penn, 1995). Therefore, molecular markers to distinguish between the two forms of molar pregnancies and hydropic abortions are of great interest.

The group of testicular germ-cell tumors (TGCT) in post-pubertal patients consists of several histologic subtypes: seminomas, embryonal carcinomas (EC), yolk sac tumors (YST), teratomas, and choriocarcinomas (CC). They are characterized by a high incidence of up to 54 % of mixed-form neoplasias (Sesterhenn, Davis Jr, 2004). The subtype of tumor is of clinical relevance (Albers *et al*., 2003; Eble *et al*., 2004; Krege *et al*., 2008). Therefore, a clinical determination of the components by a pathologist is routinely undertaken. Even though a number of antibodies is available, this identification can pose a challenge (Berney *et al*., 2012). *HMGA2* expression in TGCTs has been reported before (Franco *et al*., 2008). The authors reported moderate to high expression of *HMGA2* in ECs and YSTs using immunohistology, Western-Blot, and RT-PCR. The aim of the investigation part of this dissertation was to distinctively ascertain the expression level and test for a possible clinical application, using highly sensitive qRT-PCR and immunohistochemistry.

Virtually all studies mentioned above investigated only one of the two genes analyzed for

this thesis. There are a few studies involving both *THADA* and *HMGA2*, although never in direct connection. Voight *et al*. (2010), who confirmed the association between a *THADA* variant and T2D, also reported a new risk loci near *HMGA2* in context with the disease. Louwers *et al*. (2013) and Saxena, Welt (2013) analyzed *THADA* and *HMGA2* variants in association with PCOS. While the two studies differ in part which SNPs for *THADA* and *HMGA2* were analyzed, Louwers *et al*. (2013) reported a significant association between *THADA* variants and PCOS, while Saxena, Welt (2013) did not find such a correlation. For *HMGA2*, both groups conclude that there is no association between *HMGA2* gene variants and PCOS.

*THADA* was discovered in benign thyroid tumors. One main aspect of this thesis was to advance the knowledge about the role of this gene in this organ, especially in hyper- and neoplasias. Since an association between *THADA* and *HMGA2* became apparent in dedifferentiated thyroid tissue, focus was also directed towards this issue in other human tissues. Further studies were aimed at certain aspects of *HMGA2* in extra-embryonic tissue, namely fetal placenta, and dedifferentiated testicular tissue, through expression analysis by quantitative real-time PCR and immunohistochemistry.

# 2. Materials and methods

Detailed information about materials and methods is given in the publications II to IV. Additional content is provided in this section.

## 2.1. Establishing of *THADA*-vectors

For determination of the localization of THADA, *THADA* ORF was cloned into green fluorescent protein vectors pEGFP-C1 and -N1 (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France). Full-length *THADA-A3* PCR product was kindly provided by Norbert Drieschner (Center for Human Genetics at the University of Bremen). After A-tailing with GoTaq Flexi polymerase (Promega GmbH, Mannheim, Germany), the fragment was ligated into pGEM-T Easy (Promega), in both cases following the manufacturer's instructions. Transformation into *DH5α* (Merck Chemicals, Darmstadt, Germany) was done according to the protocol by Inoue *et al*. (1990), clones were selected after a blue/white-screening on AIX (100 µg/ml Ampicillin, 0,5 mM IPTG, 50 µg/ml X-Gal) agar plates. After over-night culture in LB (Luria-Bertani) medium, glycerol stocks were prepared by adding aliquots of 1 ml bacterial culture to 1 ml 60% (v/v) glycerol in a sterile reaction tube which were stored at -80°C. Plasmid-DNA was isolated with the Qiagen Plasmid Midi Kit and the QIAprep Spin Miniprep Kit, respectively (Qiagen, Hilden, Germany). Sequence analysis was performed by Eurofins MWG Operon (Ebersberg, Germany), for assessment of the data, Vector NTI (Invitrogen, Karlsruhe, Germany) and Chromas Lite (Technelysium Pty Ltd, South Brisbane, Australia) were used. For replacement of sections containing mutations, pre-prepared subfragments of *THADA* ORF were utilized. These plasmids were generated in identical fashion as the full length clone (see above). In addition, Pfu DNA Polymerase (Promega) was used to obtain PCR fragments, according to the manufacturer's instructions. Restriction enzymes AatII, ApaI, BamHI, BclI, BglII, Bsp119I, Bpu1102I, BshTI, BstXI, Eco32I, EcoO109I, EcoRI, Esp3I, HhaI, HindIII, Kpn2I, NcoI, NdeI, NotI, PaeI, PstI, RsaI, SalI, TaqI, TasI, and XhoI, and in addition, CIAP, T4 polymerase, and T4 ligase (all Fermentas, St. Leon-Rot, Germany), were used according to the manufacturer's instructions. When necessary, DNA purification was performed with the QIAquick PCR Purification Kit (Qiagen). To separate different DNA fragments after digestion, gel electrophoresis was applied. For this, depending on the size of the

fragments, the appropriate amount of agarose (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) was added to TAE buffer, the solution was heated until melting of the agarose. It was then poured into a sealed tray, simultaneously adding ethidium bromide (Invitrogen) to the final concentration of 150 ng/ml. After hardening, it was put into a gelelectrophoresis apparatus (Gibco, Karlsruhe, Germany), voltage was applied via a power supply unit (Gibco). Afterward, the gel was evaluated with a gelelectrophoresis documentation system (Biostep, Jahnsdorf, Germany), and the desired band was cut out by scalpel (HMD Healthcare, Hereford, Great Britain). Isolation of the DNA was performed with the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. For transformation of the Plasmid-DNA into *DH5α* and isolation after over-night culture see above. After sequencing and analysis of the data, only clones without mutations were kept. Except for the blue/white screening, the same materials and methods were also applied for construction of the following clones: *THADA-A3* in pEGFP-C1, *THADA-A3* in pEGFP-N1, *THADA-A3-Fus3p* in pEGFP-C1 and *THADA-A3-Fus3p* in pEGFP-N1, *THADA-A3-Fus3p* being an aberrant form as detected by Rippe *et al*. (2003) in thyroid adenomas.

## 2.2. Cell lines and tissue samples

In all cases, human tissue samples and cell lines derived from human tissue, respectively, were used.

### 2.2.1. Cell lines

Following cell lines were available at the Center for Human Genetics (ZHG) at the University of Bremen and were kindly provided by PD Dr. Gazanfer Belge: see table 1.

**Table 1:** Cell lines used for this thesis.

| **cell line** | **tissue** | **established by** |
|---|---|---|
| S40.2/TSV40 | thyroid adenoma | PD Dr. G. Belge, ZHG |
| S121/TSV40 | thyroid adenoma | PD Dr. G. Belge, ZHG |
| S141.2/TSV40 | thyroid adenoma | PD Dr. G. Belge, ZHG |
| S211/TSV40 | thyroid adenoma | PD Dr. G. Belge, ZHG |
| S325/TSV40 | thyroid adenoma | PD Dr. G. Belge, ZHG |

| cell line | tissue | established by |
|---|---|---|
| S533/TSV40 | thyroid adenoma | PD Dr. G. Belge, ZHG |
| FTC133 | follicular thyroid carcinoma | PD Dr. C. Schmutzler, Charité |
| FTC238 | follicular thyroid carcinoma | PD Dr. C. Schmutzler, Charité |
| HTH74 | anaplastic thyroid carcinoma | PD Dr. C. Schmutzler, Charité |
| S277 | anaplastic thyroid carcinoma | PD Dr. G. Belge, ZHG |
| Jurkat | T-cell leukemia | Dr. J. Minuwada, Fujisaki Cell Center |
| SupT1 | T-cell lymphoma/leukemia | Dr. M. Gramatzki, Uniklinik Erlangen |
| Myco 1T | pleomorphic adenoma of the salivary glands | PD Dr. G. Belge, ZHG |
| Li14/TSV40 | lipoma | PD Dr. G. Belge, ZHG |
| Fi primary cells | skin fibroblast | PD Dr. G. Belge, ZHG |

tissue: tissue (tumor type) cell line deriving from.

### 2.2.2. Snap-frozen lung samples

Lung cancer samples and the corresponding normal tissue specimens were collected at the Clinical and Experimental Pathology of the Research Center Borstel, Germany.

### 2.2.3. Blood samples

Blood samples were taken at the Central Hospital Bremen-Mitte, Bremen, Germany and at the Department of Internal Medicine, Division of Hematology and Oncology, University of Rostock, Germany.

### 2.2.4. Prostate samples

Prostate samples were collected at the National Institute of Public Health and Environmental Protection, Department of Chronic Disease and Environmental Epidemiology, Bilthoven, the Netherlands.

### 2.2.5. Snap-frozen normal tissues

Normal tissue samples (thyroid, salivary gland, lung, heart, myo- and endometrium, blood, and adipose tissue) used in the *THADA* expression study (section 3.2.) were collected under the supervision of the Center for Human Genetics of the University of Bremen, Germany.

### 2.2.6. FFPE thyroid samples

Formalin-fixed paraffin-embedded (FFPE) thyroid tissue samples were collected at the Department of Pathology, Clinical Center Bremen-Mitte, Bremen, Germany.

### 2.2.7. FFPE placenta samples

FFPE placenta tissue samples and hydatidiform moles were collected at the Institute for Pathology, Elbe Clinic Stade-Buxtehude, Germany, and the Department of Pathology, Clinical Center Bremen-Mitte, Bremen, Germany, respectively.

### 2.2.8. Testis samples

Testis FFPE tissue samples were collected at the Department of Pathology, Albertinen Hospital, Hamburg, Germany, the Department of Pathology, Clinical Center Bremen-Mitte, Bremen, Germany, and the Institute for Pathology, Elbe Clinic Stade-Buxtehude, Germany. Additional FFPE samples were collected under the supervision of the Leibniz Institute for Prevention Research and Epidemiology, Bremen, Germany. Snap-frozen normal testis samples were collected by the Department of Pathology, Clinical Center Bremen-Mitte, Bremen, Germany.

## 2.3. RNA isolation and cDNA synthesis

For snap-frozen normal tissues and adipose tissue samples, RNA was isolated using the RNeasy Mini Kit and RNeasy Lipid Tissue Mini Kit, respectively (Qiagen). For cell lines TRIzol reagent (Invitrogen) was employed. RNA isolation from FFPE blocks was performed utilizing a microtome. Depending on the size of the embedded tissue, six to eight cuts of 5 µm thickness were used, for snap-frozen specimens a TissueLyser (Qiagen) was applied. For separate investigations, different isolation kits were utilized. Roche High Pure RNA Paraffin Kit (Roche, Mannheim, Germany) was chosen for the *THADA* expression analysis in all but the placenta samples, RNeasy FFPE Kit (Qiagen) was used in the *NIS* investigation. In the remaining tests, total RNA isolations were performed using the innuPREP Micro RNA Kit (Analytik Jena AG, Jena, Germany). All

isolations were performed according to the manufacturer's instructions with the following modifications for the innuPREP Micro RNA Kit: Lysis of the paraffin sections preceding RNA isolation was conducted using TLS-Lysis solution and Proteinase K from the innuPREP DNA Micro Kit (Analytik Jena) without prior deparaffinization. Sections were incubated for 1 h at 60°C and 15 min at 80°C. Determination of RNA concentration was performed by photometer (Eppendorf, Hamburg, Germany). For blood, cell lines, lung, prostate, and part of the placenta samples, RNA isolation was performed by Inga Flor (placenta), Anke Meyer (cell lines, prostate), and Britta Meyer (blood, lung), respectively. In these cases the isolated RNA was kindly provided.

RNAs were reverse-transcribed into cDNA by M-MLV Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. In all cases, each sample contained 250 ng RNA, 200 U enzyme and 150 ng random hexamer primer (Invitrogen).

## 2.4. qRT-PCR

Real-time PCR was performed using the Applied Biosystems 7300 sequence detection system according to the TaqMan Gene Expression Assay Protocol (Applied Biosystems, Darmstadt, Germany) in 96-well microtiter plates with a total volume of 20 µl. In case of TaqMan gene expression assays for *THADA* (assay number Hs00152982, Applied Biosystems, Foster City, USA), *NIS* (assay number Hs00166567_m1, Applied Biosystems), and *HMGA2* (assay number Hs00171569, Applied Biosystems), each reaction consisted of 2 µl of cDNA reverse transcribed from 25 ng of total RNA, 10 µl of TaqMan Universal PCR Master Mix (Applied Biosystems), 1 µl of TaqMan assay and 7 µl of $ddH_2O$. For the 18S rRNA assay, using 18S forward and 18S rev_1 primers (Antonov *et al*., 2005), each reaction consisted of 2 µl of cDNA (1:10 diluted in $ddH_2O$, with regard to the higher expression of 18S rRNA in comparison to the genes above) reverse transcribed from 25 ng of total RNA, 10 µl of TaqMan Universal PCR Master Mix, 600 nM of forward and reverse primers, 200 nM of 18S probe (Antonov *et al*., 2005) and 5.4 µl of $ddH_2O$. For the *HPRT* assay, using HPRT FP and HPRT RP primers, and HPRT probe (Specht *et al.*, 2001), except for the dilution of the cDNA, the same conditions applied. Thermal cycling specifications were 2 min at 50°C followed by 10 min at 95°C, 50 cycles at 95°C for 15 s, and 60°C for 1 min. A non-template control of amplification and two negative controls of

previous cDNA synthesis (one without RNA and one missing reverse transcriptase) were included in each plate for the *NIS* and all *THADA* investigations except for placenta samples. For *HMGA2*, *HPRT*, and *THADA* in the placenta studies (normal and molar pregnancies), and for *HMGA2* and *HPRT* in the testis study, for each sample a negative control of previous cDNA synthesis (missing reverse transcriptase) and for each plate a non-template control of amplification and a non-template control of previous cDNA synthesis were included in each run. Software Sequence Detection Software 1.2.3 (Applied Biosystems) was programmed with the reaction conditions. All testing reactions were performed in triplicate. Gathered qRT-PCR data was analyzed using the comparative $C_T$-method ($\Delta\Delta C_T$-method) (Livak, Schmittgen, 2001).

## 2.5. Fluorescence in situ hybridization

Fluorescence in situ hybridization used for determinations of ploidy level of hydatidiform moles was performed by Norbert Drieschner with technical assistance by Lisa Imbil and Tais Sommerfeld. For detection of polyploidy, interphase-FISH with a probe mixture containing a 1q12-specific probe (PUC1.77) and a centromere 6-specific probe (D6Z1; pEDZ6) was performed on formalin-fixed, paraffin-embedded (FFPE) tissue sections. Labeling of probes was done by nick translation (Abbott Molecular, Wiesbaden, Germany) either with SpectrumOrange-dUTP (PUC1.77) or SpectrumGreen-dUTP (pEDZ6) (Abbott). FISH was carried out as described previously (Klemke *et al.*, 2012) with a few modifications. Digestion of 4 µm tissue sections was performed with a pepsin ready-to-use solution (DCS, Hamburg, Germany) at 37°C within a range of 30 and 45 min. The digestion time was optimized for each tissue section. 200 non-overlapping nuclei from different (at least four) areas of the tissue were finally scored, using a Zeiss Axioskop 2 Plus (Carl Zeiss Microscopy GmbH, Göttingen, Germany) microscope. Nuclei with two signals for each probe were scored as diploid, nuclei with three signals for each probe as triploid, and those with four signals for both probes as tetraploid.

## 2.6. $p57^{KIP2}$ immunohistochemical analysis

$p57^{KIP2}$-specific immunostaining was performed by Käte Burchardt on representative sections from formalin-fixed, paraffin-embedded tissue sections of all samples initially diagnosed as hydatidiform moles, using a mouse antibody against the recombinant human $p57^{KIP2}$ protein (Zytomed Systems GmbH, Berlin, Germany). For the immunohistochemistry the SuperVision 2 HRP kit was used (DCS, Hamburg, Germany). 4 µm sections from FFPE blocks were incubated on slides overnight at 56°C. The tissue sections were deparaffinized in xylene and alcohol, then blocked with 3 % $H_2O_2$ solution. After rehydration, the samples were incubated for 45 min in a steamer, using 0.01 M citrate buffer, pH 6. This took place initially at room temperature heating up for about 20 min until boiling for the rest of the duration, then cooled down for 20 min and rinsed with distilled water. Next, the slides were incubated consecutively in antibody solution (1:3000 in antibody diluent (Zytomed Systems)) for 30 min, in enhancer solution for 20 min, in HRP-polymer solution for 20 min, in DAB 2 solution for 5 min, and in hematoxylin (1:2 in distilled water) for 5 min. Between each of these steps a rinsing with wash buffer took place. Finally, the samples were treated with alcohol and xylene and afterward sealed with Pertex (Medite GmbH, Burgdorf, Germany).

Interpretation of $p57^{KIP2}$ staining was performed by a pathologist, using a Zeiss Axioskop microscope, samples were considered $p57^{KIP2}$-positive only when a distinct nuclear immunostaining of villous stromal cells and cytotrophoblasts was observed. Samples with faint nuclear staining were considered negative. Staining of intermediate trophoblasts and maternal decidua served as the positive internal control.

## 2.7. Statistical analysis

Statistical calculations were done using R (The R Foundation for Statistical Computing, Vienna, Austria) and SAS (SAS Institute Inc., Cary, North Carolina, USA).

The following analyses were utilized: The two-sided Wilcoxon rank sum test (equivalent to the Mann-Whitney U test) and the Welch t test were used to compare average values from two independent groups, respectively; relationships between two amounts were quantified by linear regression, by a nonparametric spline model, or by Spearman's rank correlation

coefficient. Statistical models with nested sets of explaining quantities were compared by the likelihood ratio test. Sensitivity, specificity and decision limits were calculated from non-parametric density estimations. A *p*-value of less than 0.05 was considered significant, a *p*-value of less than 0.001 was deemed highly significant. Analyses were performed with Dr. Werner Wosniok (Institute of Statistics, University of Bremen).

Regarding the analyses of *THADA* and *HMGA2* expression in section 3.3 (unpublished results), tests for normal distribution were performed according to Anderson-Darling, Cramer-von Mises, Kolmogorov-Smirnov, and Shapiro-Wilk. If normally distributed, differences in expression were examined by a one-way ANOVA or Welch's ANOVA (in case variances turned out to be unequal as suggested by the Bartlett's test). If not following a normal distribution, the Wilcoxon rank sum test or the Kruskal-Wallis test (in case the grouping variable contained more than two categories) were conducted. For matched specimens, the Wilcoxon signed rank test was employed. Linear relations were quantified by the Pearson correlation coefficient. Reliability of the coefficient of determination (the squared Pearson correlation) was checked by the F-test. Statistical consulting was kindly provided by Dr. Christian Astrosini.

# 3. Results

## 3.1. Characterization of thyroid adenoma associated (THADA) gene and protein

### Publication I: THADA - a protein with an important role in the thyroid (Kloth *et al.*, 2012)

Investigations by Volkhard Rippe and Norbert Drieschner at the Center for Human Genetics at the University of Bremen revealed the frequent aberration of one at that point in time unknown gene in thyroid adenomas. This gene was later named *thyroid adenoma associated* (*THADA*) (Rippe *et al*., 2003). After elucidation of its genomic structure by *in silico* analysis, the aim of this study was to determine the cellular localization of the protein.

*THADA* is located on chromosome 2, in band 2p21 and spans about 365 kbp. *In silico* analysis of expressed sequence tags (EST) revealed a number of relatively short exons in the 5'-region of the gene, bringing the total amount to 38. In this full-length form, the *THADA* ORF encodes for 1954 aa (*THADA-A1*), a splice variant without exons 27 and 28 contains 1880 aa (*THADA-A2*), a second one, missing exons 16 and 17, encompasses 1833 aa (*THADA-A3*).

To determine the localization of THADA in the cell, a plasmid containing the full-length ORF of *THADA-A3* was cloned. Splice variant *THADA-A3* was used for the experiments, since this form showed the highest expression as determined by RT-PCR on cDNA from cell cultures. Several cell lines were tested as template. As a result, MCF-7 turned out to contain a single nucleotide polymorphism (A4946G). This was not the case for S40.2, which was consequently used as the template in the PCR. This amplicon was cloned into pGEM-T Easy. In addition to this plasmid, a second one containing *THADA-A3-Fus3p*, a truncated form found in thyroid adenomas, also served as basis for the expression vectors. In separate experiments, pEGFP-C1- and pEGFP-N1-vectors each containing the full-length (without exons 16 and 17) and the truncated ORF of *THADA* were transfected into human lipoma cells. All variants containing part of or the full-length *THADA* ORF showed the same pattern, i.e. a strong signal in the cytoplasm but none in the nucleus. For the negative controls without *THADA*, the protein was evenly distributed in the cells. This investigation revealed that THADA is not a core protein and that the truncation of *THADA*

does not cause a change in the location of its protein.

# I

# THADA - a protein with an important role in the thyroid

Lars Kloth, Norbert Drieschner, Volkhard Rippe, Gazanfer Belge, Inga Dietz, Jörn Bullerdiek

Poster presentation, 55. Symposium der Deutschen Gesellschaft für Endokrinologie, Mannheim, Germany

Own contribution:

Study design with Norbert Drieschner and Jörn Bullerdiek

*In silico* analysis of 5'-*THADA* structure with Jessica Hommes

Establishing of *THADA*-vectors

Creation and presentation of the poster

Center for Human Genetics
University of Bremen

# THADA - a protein with an important role in the thyroid

**Lars Kloth[1], Norbert Drieschner[1], Volkhard Rippe[1], Gazanfer Belge[1], Inga Dietz[1], Jörn Bullerdiek[1]**

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## Discovery of *THADA* and elucidation of its genomic structure

Hyperplasias of follicular epithelial origin and benign thyroid tumors have been cytogenetically analyzed comprehensively. One in five of these lesions showed cytogenetic aberrations, translocations involving chromosomal band 2p21 were found to be the second most frequent structural chromosomal rearrangement. *Thyroid adenoma associated (THADA)* was identified as the target gene affected by these translocations and the concomitant truncation of the gene is speculated to be the critical event for the development of the tumors[1]. The full length cDNA of *THADA* consists of 6,134 bp and contains 38 exons [GenBank: NM_022065]. There are two known splice-variants, one without exons 16 and 17, one lacking exons 27 and 28 (fig. 1). In each of the aforementioned translocations *THADA* was truncated after exon 28 or 26, respectively and ectopic sequences were fused to it.

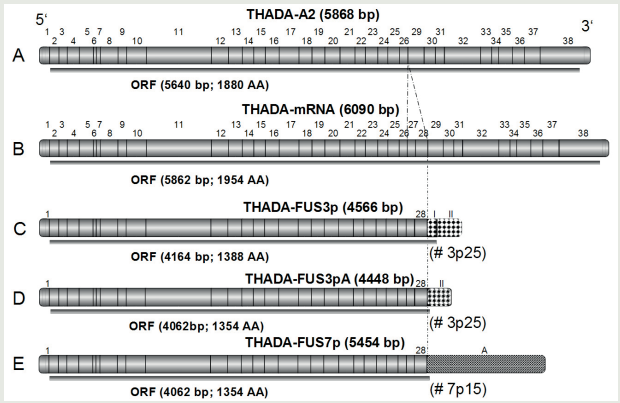


Figure 1: Structure of *THADA-A2* (A), *THADA* (B), *THADA-Fus3p* (C, D), and *THADA-Fus7p* (E) mRNA.

## Localization of the protein in the cell

Using the Green-Fluorescence-Protein (GFP) vector system, THADA-A3 (deriving from the alternative splice variant without exons 16 and 17) as well as THADA-A3-Fus3p were detected in the cytoplasm but not the nucleus (fig. 2). Negative-controls without an insert showed a concomitance in the whole cell. GFP-C1 as well as GFP-N1 vectors were used in all cases. Later, the results were confirmed by Western-blot analysis. These findings suggest that the truncated 3'-part is of no importance for the cellular localization of the protein and that there is no relation between the altered function of the truncated protein and its localization.

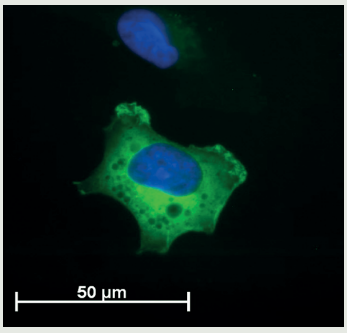


Figure 2: Localization of THADA in the cytoplasm but not the nucleus of Li-14-cells after transfection of pEGFP-C1-THADA-A3; DAPI counter-staining of the nuclei.

## *THADA* in other species

Comparison of *THADA* homologous in *Homo sapiens*, *Chlorocebus aethiops*, *Canis familiaris*, *Mus musculus*, and *Gallus gallus* revealed that the gene consist of 38 exons throughout the species[2]. In all organisms the startcodon was found to be located in exon 2, the stopcodon in exon 38. The genomic length varied considerably but the open reading frame only slightly, the latter consisted from 5793 bp (*Gallus gallus*) to 5862 bp (*Homo sapiens* and *Chlorocebus aethiopis*). A highly conserved region (aa 1033-1415 in *Homo sapiens*) was discovered, this part of the protein gets disrupted when *THADA* becomes truncated as described in human thyroid adenomas. Additionally, a homology to a protein-protein-interaction-domain of the superfamily ARM repeat in THADA was reported.

## Expression of *THADA* in normal tissues

In an investigation testing eight different healthy tissues via qRT-PCR, *THADA* was expressed significantly higher in thyroid samples than in those of salivary gland, lung, heart, myo- and endometrium, blood, and adipose tissue[3] ($p < 0.00001$, exact Wilcoxon signed rank test) (fig. 3).

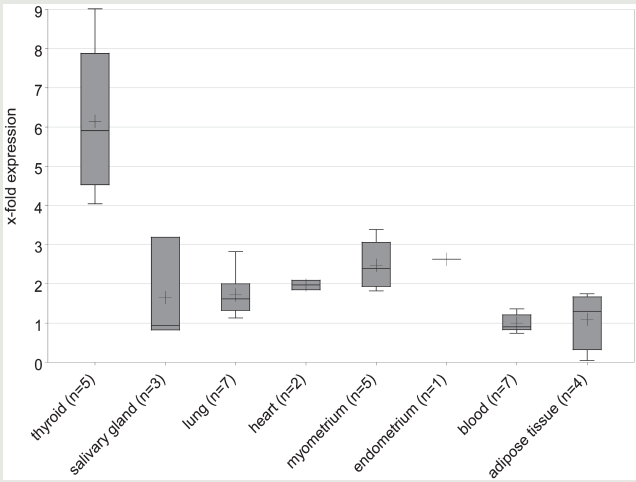


Figure 3: Boxplots for the expression of *THADA* (relative quantification) in normal tissues, tissue type at x-axis. (n: number of samples)

## *THADA* expression as a marker of differentiation of the thyroid

By testing thyroid lesions the level of *THADA* expression turned out to be a marker for the dedifferentiation of thyroid tissue[3]. Eight normal tissues, 18 goiters, 35 benign, and 32 malignant tumors were measured, distinct differences were found especially comparing the group of anaplastic carcinomas (ATCs), the most dedifferentiated type of malignant tumor, with other lesions. The ATCs showed significantly lower levels of expression. Overall, the expression level was found to be correlated with *NIS* (*sodium-iodide symporter*) and negatively correlated with *HMGA2* (*High-mobility group AT-hook 2*), both established markers for the differentiation of the thyroid. In another investigation using Western-Blot, in ATC cell lines FRO, HTH-74 and S277 no THADA was detectable as opposed to cell lines deriving from other malignant and benign thyroid tumors, goiter and normal thyroid tissue.

## Conclusion

Since its discovery as the target gene affected by certain translocations in thyroid adenomas new information about *THADA* has become available, hinting at a crucial role in the thyroid.

## References

[1] Rippe *et al*., Oncogene 2003, 22:6111-6114.

[2] Drieschner *et al*., Gene 2007, 403:110-117.

[3] Kloth *et al*., BMC Clin Pathol. 2011, 11:13.

## 3.2. Quantitative analysis of *THADA* expression in thyroid lesions and normal tissues

**Publication II: Decrease in thyroid adenoma associated (*THADA*) expression is a marker of dedifferentiation of thyroid tissue (Kloth *et al*., 2011)**

2p21 aberrations are one of the main chromosomal changes in thyroid adenomas. Rippe *et al*. (2003) showed that these aberrations cause the truncation of *THADA*. Therefore, an important role of the gene in the thyroid seemed conceivable. To test this hypothesis, *THADA* expression was measured in this organ and the values gathered were compared to those of other normal tissues, using qRT-PCR. All thyroid samples showed a higher expression than any of the other specimens. Overall, the level was significantly above those of salivary gland, lung, heart, myo- and endometrium, blood, and adipose tissue.

In addition to the normal tissue samples *THADA* expression was also measured in thyroid hyper- and neoplasias. Eight normal tissue samples, 18 goiters, 35 benign tumors (including three specimens with a 2p21 translocation), and 32 malignant tumors (19 papillary carcinomas, five follicular carcinomas, four medullary carcinomas, four anaplastic carcinomas) were tested. Samples with a 2p21 aberration showed only slight deviations from the healthy tissue samples. Opposite to the differentiated thyroid adenomas, for the highly dedifferentiated anaplastic carcinomas, the level of expression was significantly lower, indicating an involvement of *THADA* in the differentiation in thyroid cells.

Belge *et al.* (2008) found *HMGA2* to be a marker of differentiation in thyroid tissue, applying qRT-PCR on part of the same samples used in this investigation. Comparing the expression values of both genes revealed a significant correlation of *THADA* and *HMGA2*. An increase in *HMGA2* is associated with a decrease in *THADA* expression. Decreased *THADA* expression as a marker for dedifferentiated thyroid tissue was further verified by a significant correlation between *THADA* and *NIS* (*sodium-iodide symporter*), a well described marker of differentiation in the thyroid.

## II

# Decrease in *thyroid adenoma associated* (*THADA*) expression is a marker of dedifferentiation of thyroid tissue

Lars Kloth, Gazanfer Belge, Käte Burchardt, Siegfried Loeschke, Werner Wosniok, Xin Fu, Rolf Nimzyk, Salah A. Mohamed, Norbert Drieschner, Volkhard Rippe and Jörn Bullerdiek

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Own contribution:

Study design with Jörn Bullerdiek

Execution and analysis of the study except for bioinformatics

Statistics with Werner Wosniok

Writing of the manuscript

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RESEARCH ARTICLE Open Access

# Decrease in *thyroid adenoma associated (THADA)* expression is a marker of dedifferentiation of thyroid tissue

Lars Kloth[1], Gazanfer Belge[1], Käte Burchardt[2], Siegfried Loeschke[1], Werner Wosniok[3], Xin Fu[1], Rolf Nimzyk[1], Salah A Mohamed[4], Norbert Drieschner[1], Volkhard Rippe[1] and Jörn Bullerdiek[1*]

## Abstract

**Background:** *Thyroid adenoma associated (THADA)* has been identified as the target gene affected by chromosome 2p21 translocations in thyroid adenomas, but the role of THADA in the thyroid is still elusive. The aim of this study was to quantify *THADA* gene expression in normal tissues and in thyroid hyper- and neoplasias, using real-time PCR.

**Methods:** For the analysis *THADA* and 18S rRNA gene expression assays were performed on 34 normal tissue samples, including thyroid, salivary gland, heart, endometrium, myometrium, lung, blood, and adipose tissue as well as on 85 thyroid hyper- and neoplasias, including three adenomas with a 2p21 translocation. In addition, *NIS* (*sodium-iodide symporter*) gene expression was measured on 34 of the pathological thyroid samples.

**Results:** Results illustrated that *THADA* expression in normal thyroid tissue was significantly higher ($p < 0.0001$, exact Wilcoxon test) than in the other tissues. Significant differences were also found between non-malignant pathological thyroid samples (goiters and adenomas) and malignant tumors ($p < 0.001$, Wilcoxon test, t approximation), anaplastic carcinomas (ATCs) and all other samples and also between ATCs and all other malignant tumors ($p < 0.05$, Wilcoxon test, t approximation). Furthermore, in thyroid tumors *THADA* mRNA expression was found to be inversely correlated with *HMGA2* mRNA. *HMGA2* expression was recently identified as a marker revealing malignant transformation of thyroid follicular tumors. A correlation between *THADA* and *NIS* has also been found in thyroid normal tissue and malignant tumors.

**Conclusions:** The results suggest *THADA* being a marker of dedifferentiation of thyroid tissue.

## Background

Benign thyroid tumors and hyperplasias of follicular epithelial origin belong to the cytogenetically best analyzed human epithelial tumors.

Cytogenetic aberrations have been detected in approximately 20% of these lesions [1]. Translocations of chromosomal band 2p21 are the second most frequent structural chromosomal rearrangement, representing a particular cytogenetic subgroup [2]. The target gene has been identified and referred to as *thyroid adenoma associated* (*THADA*) [3].

The full length cDNA of *THADA* consists of 6,134 bp distributed over 38 exons [GenBank: NM_022065]. There are two splice-variants, one lacking exons 27 and 28 [3], and the other without exons 16 and 17. The THADA protein has three isoforms corresponding to the three different transcript variants with 1953 [GenBank: NP_071348], 1879, and 1832 amino acids, respectively. In adenomas with 2p21 translocations Rippe *et al.* found different types of fusion variants of *THADA* [3]. In each case, *THADA* was truncated after exon 28 and ectopic sequences fused to it were not correlated to any known gene. Thus, it has been speculated that the truncation rather than the fusion to ectopic coding sequences is the critical event for the development of the tumor [3].

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Studies by Drieschner *et al*. [4] revealed that the mRNA, the protein size, and the genomic organization is conserved among *Homo sapiens*, *Canis familiaris*, *Chlorocebus aethiops*, *Gallus gallus*, and *Mus musculus*. THADA proteins from the analyzed organisms showed significant assignments to the superfamily ARM repeat (SSF48371; Hidden Markov Models Superfamily database), indicating the presence of a protein-protein-interaction-domain of that type.

The exact function of THADA still remains unclear. Hypothetically, it belongs to the death receptor-interacting proteins and is assumed to bind to death receptor DR5 (Puduvalli VK and Ridgway L, GenBank accession reference note), involving it in the TRAIL-induced apoptosis. The truncated THADA derived from the rearranged allele might compete with the gene product of the normal allele thereby disturbing normal apoptosis of follicular cells, and subsequently altering the steady state between proliferation and cellular death leading to adenomatous growth in benign thyroid tumors with 2p21 translocations [3]. Nevertheless, there is a need for further studies elucidating the role of THADA in normal thyroid development and in tumorigenesis.

Recently, a *THADA* variant has also been linked to type 2 diabetes (T2D) [5], but this association has not been confirmed by the majority of further studies [6-20]. During a meta-analysis of three genome-wide association studies with individuals of European descent Zeggini *et al*. found evidence for an association of a SNP (rs7578597) in exon 24 of *THADA* and the susceptibility for T2D [5]. Further indication for a correlation between *THADA* and T2D was presented in several other publications [11,14,16,17,19], one reported an altered expression of *THADA* in pancreatic islets, using data from the Diabetes Genome Anatomy Project (DGAP) database [11]. In other investigations no correlation was detected [6-8,10,12,13,15,18,20], except for one publication [9], which reported an association between *THADA* SNP rs7578597 and a 2-h insulin level during an oral glucose tolerance test but no significant association between the *THADA* SNP and T2D risk, rendering the association disputable.

The aim of this study was to analyze *THADA* expression in thyroid tissue in comparison to other tissues and to thyroid hyper- and neoplasias to elucidate the possible correlation of *THADA* mRNA with thyroid differentiation and neoplastic growth.

## Methods

### Tissue specimen and RNA isolation

RNA from snap-frozen tissues was isolated using the RNeasy Mini Kit and RNeasy Lipid Tissue Mini Kit for the adipose tissue samples, respectively (QIAGEN, Hilden, Germany).

For the formalin-fixed paraffin-embedded (FFPE) tissues of thyroid tumors, histopathologic diagnoses were performed according to the World Health Organization Classification of Tumours [21] (table 1). As to RNA isolation, FFPE blocks were cut into six sections of 5 μm for each sample using a microtome. Total RNA isolations were performed using the Roche High Pure RNA Paraffin Kit (Roche, Mannheim, Germany) for the *THADA* expression investigation and the RNeasy FFPE Kit (QIAGEN, Hilden, Germany) for the *NIS* expression analysis. Three samples were cytogenetically characterized by 2p21 translocations. In all three cases, two of which published previously [22,23], the breakpoints were narrowed down to the *THADA* locus. One of the anaplastic thyroid samples served as the source of a newly established cell line. Cytogenetical analysis revealed a highly complex karyotype with a range of 80 to 117 chromosomes (100.8 on average). Several marker chromosomes, telomeric associations, and double minutes were detected.

### cDNA-synthesis and real-time PCR expression analysis

RNAs were reverse-transcribed into cDNA by M-MLV Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). Real-time PCR was performed using the Applied Biosystems 7300 sequence detection system according to TaqMan Gene Expression Assay Protocol (Applied Biosystems, Darmstadt, Germany) in 96-well microtiter plates with a total volume of 20 μl. In case of TaqMan gene expression assay of *THADA* (assay number Hs00152982, Applied Biosystems, Foster City, USA), targeting exons 31-32, and of *NIS* (assay number Hs00166567_m1), each reaction consisted of 2 μl of cDNA reverse transcribed from 25 ng of total RNA, 10 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μl of TaqMan assay and 7 μl of $ddH_2O$. For the 18S rRNA assay, using 18S forward and 18S rev_1 primers [24], each reaction consisted of 2 μl of cDNA (1:10 diluted, with regard to higher expression of 18S rRNA) reverse transcribed from 25 ng of total RNA, 10 μl of TaqMan Universal PCR Master Mix, 600 nM of forward and reverse primers, 200 nM of 18S probe [24] and 5.4 μl of $ddH_2O$.

Thermal cycling conditions were 2 min at 50°C followed by 10 min at 95°C, 50 cycles at 95°C for 15 s and 60°C for 1 min. A non-template control of amplification and two previous negative controls of cDNA synthesis (one without RNA and one missing Reverse Transcriptase) were included in each plate. Software Sequence Detection Software 1.2.3 (Applied Biosystems) was programmed with the reaction condition. All testing reactions were performed in triplicate.

Serial dilutions were made using cDNA derived from 25, 5, 1, 0.2, and 0.04 ng of total RNA from FFPE tissue

**Table 1 Histology of the malignant thyroid lesions.**

| case no. | age (years) | sex | histology | tumor diameter (cm) | TNM classification and grading |
|---|---|---|---|---|---|
| 1 | 57 | f | PTC | 0.9 | pT1 |
| 2 | 31 | m | PTC | 2.5 | pT2 pN0 |
| 3 | 30 | f | PTC | 2.5 | pT2 NX |
| 4 | 85 | m | PTC | 4.0 | pT3a |
| 5 | 31 | m | PTC | 2.0 | pT3 pN1 |
| 6 | 54 | f | PTC | 0.6 | pT1 pNX pMX |
| 7 | 49 | f | PTC | 1.2 | pT2 |
| 8 | 38 | f | PTC | 0.6 | pT1 |
| 9 | 50 | f | PTC | 2.2 | pT2 |
| 10 | 21 | f | PTC | 1.0 | pT1 pNX pMX |
| 11 | 38 | m | PTC | 0.8 | pT1; G1 |
| 12 | 34 | f | PTC | 2.3 | pT2 pN1 pMX |
| 13 | 66 | f | PTC | 2.0 | pT3; G2 |
| 14 | 25 | f | PTC | 2.3 | pT2 pN0 |
| 15 | 42 | m | PTC | 0.7 | pT1 N0 MX |
| 16 | 42 | f | PTC | 1.4 | pT2a; G2 |
| 17 | 72 | f | PTC | 1.0 | pT1 |
| 18 | 84 | f | PTC | 6.0 | pT3 pNX |
| 19 | 27 | m | PTC | 2.5 | pT2 |
| 20 | 35 | f | FTC | 2.1 | pT2 pN0 MX |
| 21 | 66 | f | FTC | 2.0 | pT1 |
| 22 | 67 | m | FTC | 5.5 | pT3 pNX pM1 |
| 23 | 61 | m | FTC | 8.0 | pT4 |
| 24 | 53 | f | FTC | | pT4 pN1 |
| 25 | 61 | m | MTC | 3.5 | pT2 pN0 |
| 26 | 61 | m | MTC | 1.7 | pT2 |
| 27 | 52 | m | MTC | 3.3 | pT2 |
| 28 | 55 | f | MTC | 2.2 | pT2 |
| 29 | 76 | f | ATC | 1.7 | pT4b |
| 30 | 76 | f | ATC | 3.8 | pT4b |
| 31 | 86 | f | ATC | 9.0 | pT4 pN1b pM1 |
| 32 | 65 | f | ATC | 2.0 | pT4 N0; G4 |

All listed samples were used for the *THADA* expression investigation, for the *NIS* expression analysis samples 2, 3, 7, 13 and 24-28 were omitted. (PTC: papillary thyroid carcinoma; FTC: follicular thyroid carcinoma; MTC: medullary thyroid carcinoma; ATC: anaplastic thyroid carcinoma)

of one thyroid adenoma for *THADA* and 18S rRNA, and from fresh frozen tissue of one normal thyroid sample for *NIS*. In each dilution, *THADA*, *NIS*, and 18S rRNA gene expression assays were performed using absolute quantification. Afterwards, the standard curves for both assays were plotted with the log ng of input cDNA for each dilution on the x-axis, and the matched $C_T$ value on the y-axis. Furthermore, in order to evaluate the differences of amplification efficiencies, the difference of two curve slopes was calculated. If the absolute difference of the slopes is less than 0.1, the amplification efficiencies of two assays are considered to be equal and the comparative $C_T$ method is valid (User Bulletin No. 2, ABI PRISM 7700 Sequence Detection System, Applied Biosystems). 18S rRNA was used as endogenous control as suggested previously [25-28]. The 18S rRNA assay showed an amplification efficiency of 92.6% (slope = -3.514, $R^2$ = 0.995). The *THADA* assay had an amplification efficiency of 92.0% (slope = -3.531) and an $R^2$-value of 0.96. For *NIS*, the amplification efficiency was 93.4% (slope = -3.4917), the coefficient of determination amounted to 0.997). As recommended for FFPE samples [24,29-31] the fragment sizes amplified by all three assays were small, ranging between 60 and 78 bp, a validation of these values was performed via gelelectrophoresis of the PCR-products (data not shown). When applying the comparative $C_T$ method, one histological normal thyroid tissue was used as calibrator sample. Afterwards, data were compared with results from conventional histology.

For statistical analysis, the Wilcoxon signed rank test was used to compare average values (two-sided, exact

version for at most 40 cases involved, otherwise using the t approximation); relationships were quantified by linear regression and Spearman's rank correlation coefficient. Sensitivity, specificity and decision limits were calculated from non-parametric density estimations. Therefore, sensitivity and specificity may differ from raw empirical values and decision limits need not coincide with measured values. A $p$-value of less than 0.05 was considered significant.

### Ethics Statement

The use of human thyroid samples for this study was approved by the local medical ethics committee (Ethikkommission bei der Ärztekammer Bremen) and followed the guidelines of the declaration of Helsinki. Only samples that were initially taken for diagnostic purposes were secondarily used for the present study. During pathological examination, a sample of the tissue was snap-frozen. The procedure was approved by the local ethics committee. Because the samples were deidentified and were considered as samples normally discarded, the committee felt that there was no specific patient consent necessary.

As for the normal tissue samples, these were anonymously collected for earlier studies, each following the guidelines of the declaration of Helsinki.

## Results

### *THADA* expression in normal tissues

Thirty-four snap-frozen samples from eight different tissues were tested for the level of *THADA* expression.

The mean level per tissue type ranged from 1 (blood) to 6.14 (thyroid), and the lowest single value for a thyroid sample (4.04) was above the highest one (3.39, myometrium) from any of the other tissues (Figure 1).

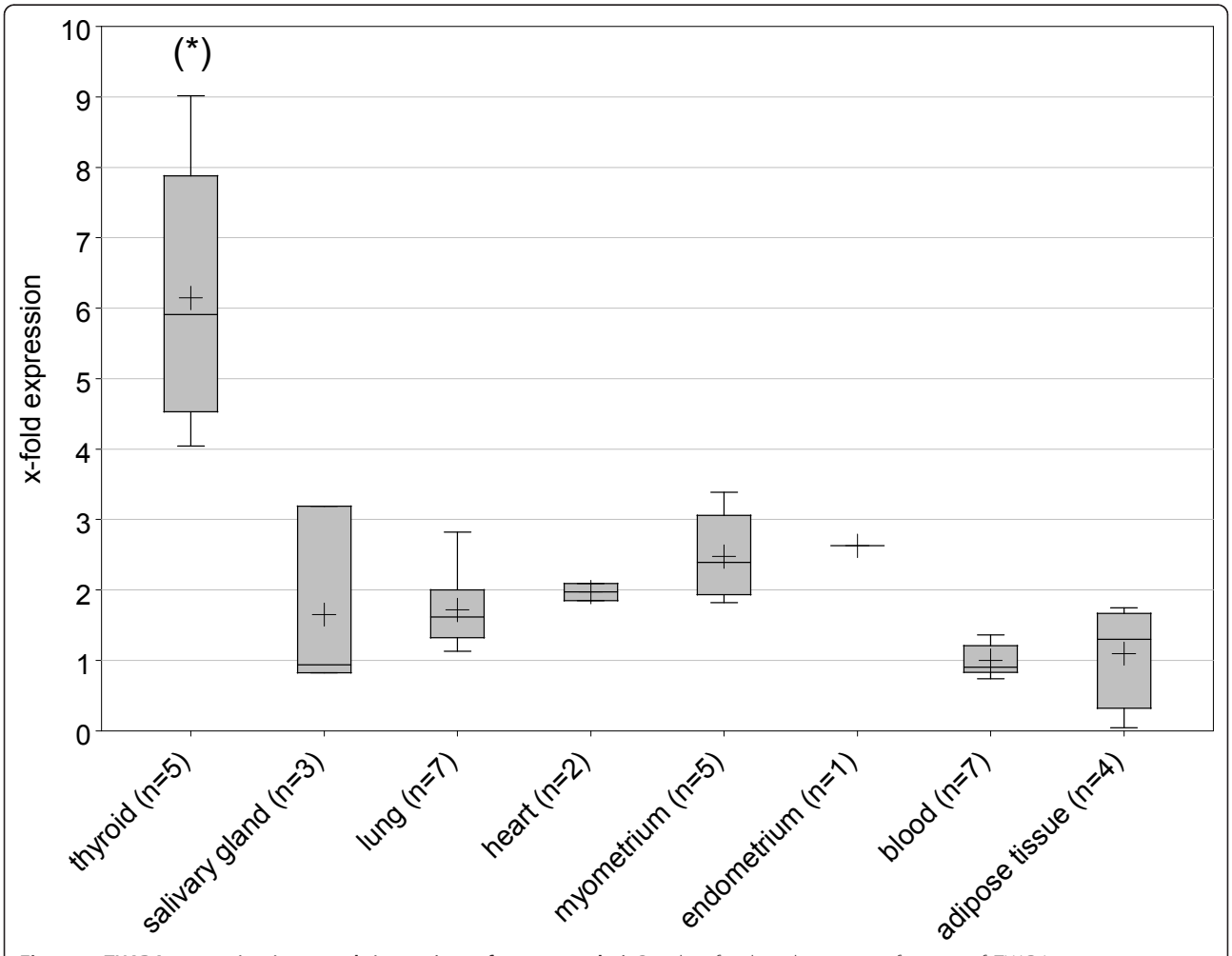


**Figure 1 *THADA* expression in normal tissues (snap-frozen samples)**. Boxplots for the relative quantifications of *THADA* gene expression in normal tissues; tissue type at x-axis. (*): $p < 0.0001$ compared to all other tissues jointly (exact Wilcoxon signed rank test). Boxes contain the inner 50% of all values and a bar at the position of the median, whiskers extend to the extrema of values or to 1.5 * box height, whichever is smaller. The plus sign shows the arithmetic mean. (n: number of samples).

Accordingly, statistical analysis using Wilcoxon's exact signed rank showed significant differences between normal thyroid tissues and the group of all other tissues ($p < 0.0001$). Using the *THADA* expression to discriminate between thyroid and non-thyroid tissue, a sensitivity of 82.5%, a specificity of 97.4% and an efficiency of 95.2% with a decision limit value of 4.23 were achieved.

### *THADA* expression in thyroid tumors

Ninety-three formalin-fixed-paraffin-embedded thyroid samples, including eight normal tissues (from four patients), 18 goiters, 35 benign, and 32 malignant tumors were measured. For single tumor samples the expression ranged between 0.065 (anaplastic carcinoma) and 2.986 (follicular adenoma) in relation to normal tissue, i.e. a ratio of 1 : 45.94. Samples with a 2p21 translocation showed a level of expression of 1.123, 1.624, and 0.662 fold, respectively. The mean values for the different tumor entities ranged from 0.423 (anaplastic carcinoma) to 1.156 (adenoma) (Figure 2 and table 2).

Significant differences of *THADA* expression were noted between benign and malignant thyroid lesions. Wilcoxon's signed rank test showed a highly significant difference comparing the joint group of goiters and benign tumors with malignant tumors ($p = 0.0009$).

Using the exact Wilcoxon test, no significant differences were detected comparing the level of *THADA* expression between normal tissue and benign lesions ($p = 0.2802$) and papillary carcinomas ($p = 0.2170$). In contrast, significant differences were found between anaplastic carcinomas (ATCs), the most dedifferentiated

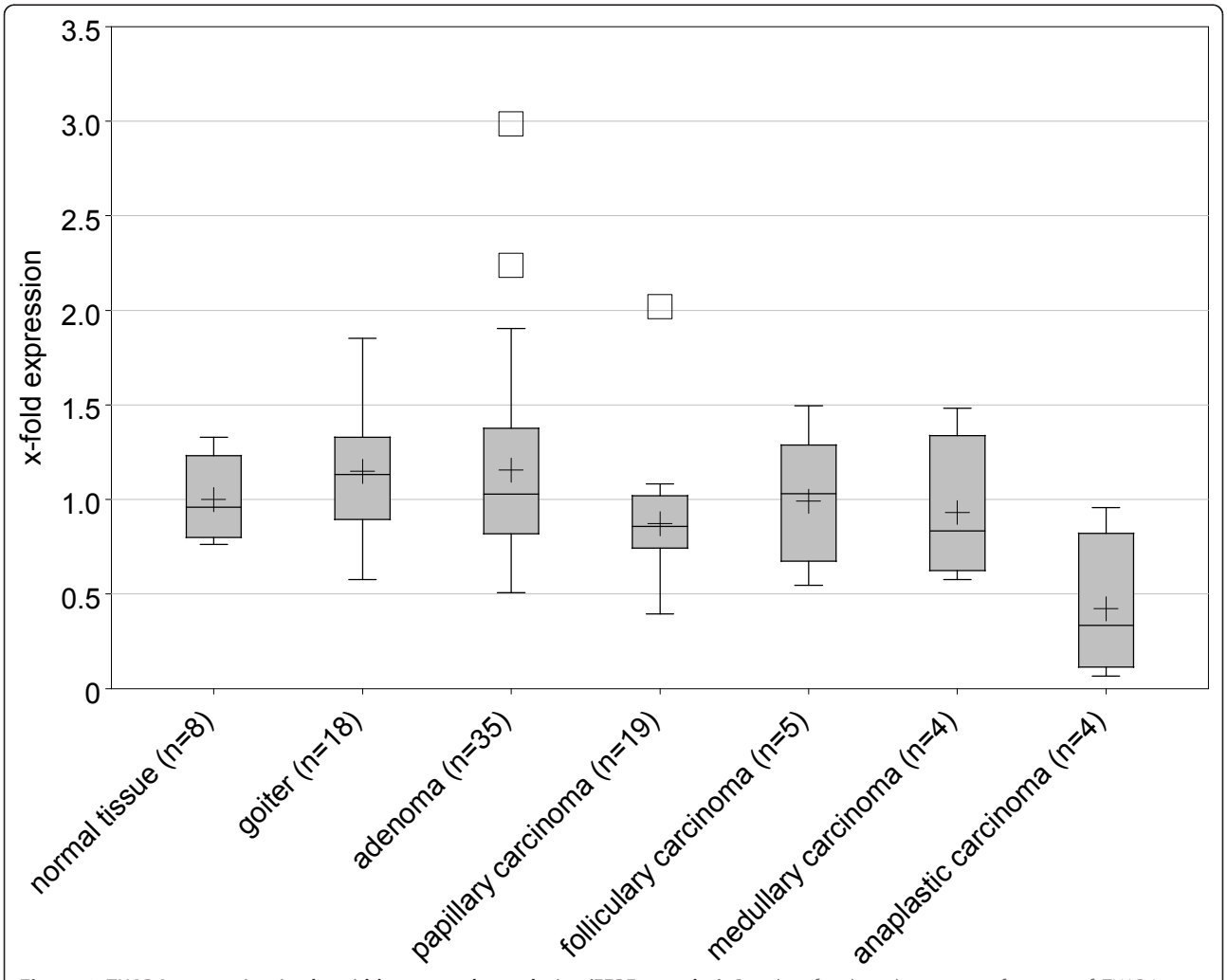


**Figure 2 *THADA* expression in thyroid hyper- and neoplasias (FFPE samples)**. Boxplots for the relative quantifications of *THADA* gene expression in thyroid normal tissue, goiter, benign and malignant tumors; normal tissue and hyper-/neoplasia type at x-axis. Boxes contain the inner 50% of all values and a bar at the position of the median, whiskers extend to the extrema of values or to 1.5 * box height, whichever is smaller, isolated symbols indicate values outside this range. The plus sign shows the arithmetic mean. (n: number of samples).

**Table 2 Detailed view of *THADA* expression in thyroid hyper- and neoplasias**

| sample type | n | average | standard deviation | median |
|---|---|---|---|---|
| normal tissue | 8 | 1 | 0.217 | 0.959 |
| goiter | 18 | 1.15 | 0.303 | 1.132 |
| *nodular goiter* | 7 | 1.266 | 0.175 | 1.305 |
| *Graves disease* | 1 | 1.103 | - | 1.103 |
| *adenomatous goiter* | 10 | 1.073 | 0.369 | 1.021 |
| adenoma | 35 | 1.156 | 0.496 | 1.029 |
| *autonomous adenoma* | 2 | 0.873 | 0.212 | 0.873 |
| *follicular adenoma* | 27 | 1.158 | 0.522 | 1.029 |
| *macrofollicular adenoma* | 1 | 1.904 | - | 1.904 |
| *microfollicular adenoma* | 4 | 1.225 | 0.268 | 1.199 |
| *oncocytic adenoma* | 1 | 0.637 | - | 0.637 |
| carcinoma | 32 | 0.842 | 0.381 | 0.842 |
| *papillary carcinoma* | 19 | 0.872 | 0.352 | 0.858 |
| *follicular carcinoma* | 5 | 0.991 | 0.353 | 1.031 |
| *medullary carcinoma* | 4 | 0.932 | 0.391 | 0.834 |
| *anaplastic carcinoma* | 4 | 0.423 | 0.383 | 0.334 |

The arithmetic mean (with the standard deviation) and median relative quantification of *THADA* gene expression in thyroid normal tissue, hyper- and neoplasias are listed. (n: number of samples)

type of thyroid tumors, and all other samples ($p$ = 0.0107) and ATCs and all other malignant tumors ($p$ = 0.0234). Comparing anaplastic carcinomas with each single group, the difference in expression between ATCs and goiters ($p$ = 0.0049) and adenomas ($p$ = 0.0058) were marked as significant. As this finding was a result of systematically comparing anaplastic carcinomas with the other lesions, a Bonferroni correction for multiple testing was used (corrected $\alpha$ = 0.0083). Without the need of correcting for multiple testing also normal tissue and papillary carcinoma would have been assessed as significantly different from anaplastic carcinoma ($p$ = 0.0485 and $p$ = 0.0350, respectively). Overall, significant results were mostly seen with the group of anaplastic carcinomas, indicating a relative stable level of expression in comparatively differentiated tissues with a significant reduction only in dedifferentiated tissues.

Recently *HMGA2* expression has been shown to indicate thyroid malignancy and can thus be considered marking the dedifferentiation of thyroid epithelium [32-34]. As to the study by Belge *et al*. [32] and the present one 48 samples were identical in both studies (seven normal tissues, one goiter, 15 adenomas and 25 carcinomas, including three anaplastic carcinomas). For these, RNA was isolated from adjacent cuts of the same FFPE block and, except for the different qRT-PCR assays, all samples were treated identical in both investigations. Thus, it was feasible to check these samples for a possible correlation between *THADA* and *HMGA2*. Using Spearman's rank correlation, there was a highly significant inverse correlation between *THADA* and *HMGA2* expression (correlation coefficient = -0.452; $p$ = 0.0015), further underlining a possible role of *THADA* in thyroid differentiation.

NIS (sodium-iodide symporter), the transmembrane glycoprotein accountable for the uptake of iodine in thyroid cells, was found to be a marker of thyroid differentiation [35-38]. To validate our findings *NIS* expression was measured in 41 samples, including seven normal tissue samples, six nodular goiters, five adenomas, and 23 carcinomas (15 papillary, four follicular, and all four anaplastic thyroid carcinomas). Using Spearman's rank correlation, no significant correlation ($p$ = 0.1288) was detected comparing *THADA* and *NIS* expression from all samples. By contrast, a significant correlation was found constraining the analysis to the follicular and papillary carcinoma samples ($p$ = 0.0497, r = 0.456, n = 19), an even stronger correlation between the expression of *THADA* and *NIS* was found in normal and all malignant samples ($p$ = 0.0021, r = 0.540, n = 30), and in normal tissue and anaplastic carcinomas ($p$ = 0.0128, r = 0.718, n = 11)

### Transcription factors binding to *THADA*

Using the SABiosciene DECODE Transcription Factor Search, no *THADA*-promotor binding sites for thyroid-specific transcription factors paired box gene 8 (pax8), thyroid transcription factor 1 (TTF1), also known as NK2 homeobox 1 (NKX2-1), and thyroid transcription factor (TTF-2), sometimes referred to as forkhead box protein E1 (FOXE1), were found. Amongst others cAMP response element-binding protein (CREB), activating transcription factor (ATF-2), c-Jun, hepatic leukemia factor (Hlf), and germ cell nuclear factor (GCNF) were marked as relevant, FOXC1, Nkx2-2, Nkx2-5, and Nkx6-1 were displayed with low relevance (data not shown). *HHEX* (hematopoietically expressed homeobox) has been found to be expressed in the adult thyroid gland and in differentiated thyroid cell lines and to be correlated with thyroid differentiation [39-41], but is not included in the SABiosciene DECODE Transcription Factor Search. A manual search for this transcription factor revealed no assured binding sites in the *THADA* promoter.

## Discussion

In this study, *THADA* turned out to be highly expressed in the thyroid compared to other normal tissues. In a group of eight different types of tissue thyroid samples showed a significantly higher *THADA* mRNA expression than salivary gland, lung, heart, myometrium, endometrium, blood, and adipose tissue, hinting at a possibly important role of *THADA* in the thyroid.

The results in part contradict data available online. NCBI ESTProfileViewer predicted a higher expression in heart and lung tissue and a slightly lower in the thyroid. For uterus and blood the data are in concordance with

those obtained from the EST-based estimates. For salivary gland and adipose tissue the TPM (transcripts per million)-values are zero, this could be due to an overall small EST pool (20155 ESTs for salivary gland, 13106 ESTs for adipose tissue), resulting in less than one gene EST (all normal tissues average: 31073 ESTs per gene EST). Comparison to Affymetrix GeneChip Human Genome array-based results from The Genomics Institute of the Novartis Research Foundation (GNF) showed similar discrepancies. There are three probes, one (gnf1h10751_at) is diverging considerably from the other two and was therefore omitted. Compared to our data both remaining probes resulted in similarly average Spearman's rank correlation coefficients and no significances ($p \geq 0.2$). GNF results showed thyroid as the tissue with the highest *THADA* expression but less distinct from the other tissues. Overall, the more precise and reliable qRT-PCR-method disclosed results that are diverging from those available from online databases.

Furthermore, evidence that *THADA* expression is associated to thyroid differentiation has been presented. Analysis of 93 thyroid FFPE samples revealed significant differences between benign and malignant thyroid lesions, especially when comparing the group of anaplastic carcinomas with other types of lesions. Despite one outlier with an expression level almost identical to normal tissue, the values were significantly lower compared to all other samples as well as to all other malignant tumors. A comparison of the expression level of *THADA* and *NIS* (*sodium-iodide symporter*) confirmed these observations. Amongst others, a significant correlation between *THADA* and this well established marker of thyroid differentiation [35-38] has been detected in normal tissue and anaplastic carcinomas. This suggests that *THADA* expression decreases with dedifferentiation of the thyroid epithelium. This hypothesis is further supported by the significant inverse correlation between the expression of *THADA* and *HMGA2*. Belge *et al.* [32] showed that *HMGA2* is significantly overexpressed in malignant thyroid tumors compared to benign lesions. As a rule, a high *HMGA2* expression seems to be accompanied by a low *THADA* expression. As yet the underlying mechanism is unknown but it does not seem to involve thyroid-specific transcription factors, since no binding sites for pax8, TTF-1 and -2 were found. However, the SABiosciene DECODE Transcription Factor Search revealed a binding site of the cAMP response element-binding protein (CREB). CREB has been shown to regulate diverse cellular responses, including differentiation [42], targeted expression of dominant-negative mutants of CREB in transgenic mice has been associated with thyroid hypoplasia [43]. cAMP indirectly plays a crucial role in the differentiation of endocrine tissues [43], including the thyroid [44,45]. Thus one might speculate about an involvement in the decreased expression of *THADA* in dedifferentiated thyroid cells.

In thyroid adenomas *THADA* was frequently found to be truncated [3]. Whereas the intact *THADA* may be involved in maintaining the differentiation of thyroid epithelium, the truncated allele might play a key role in tumor development of the thyroid. While competing with the full-length protein translated from the normal allele of *THADA* the altered protein derived from the truncated gene might lead to an impaired induction of apoptosis, and subsequently give rise to an increased cell proliferation leading to benign thyroid tumors with 2p21 translocations [3], without significant changes of the expression level.

## Conclusions

*THADA* expression, though not restricted to the follicular cells of the thyroid, is higher in the thyroid than in other tissues tested (salivary gland, heart, endometrium, myometrium, lung, blood, and adipose tissue). As to its normal function, *THADA* expression has been found to be decreased in anaplastic carcinomas and to be correlated with the expression of *NIS*, a marker of thyroid differentiation, and inversely correlated with that of *HMGA2*, a marker of malignant transformation of the thyroid and cancer stemness. It may thus have essential functions in maintaining the differentiation of the follicular epithelium.

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#### Authors' contributions

LK conceived and designed the study, carried out the molecular genetic studies, took part in the statistical analysis and the search for transcription factors and drafted the manuscript. GB provided the study material (FFPE and part of the snap-frozen samples), and revised the manuscript. KB carried out the clinical workout and performed the pathological analysis. SL performed the pathological determination of the samples (verification). WW carried out the statistical analysis. XF took part in the molecular studies. RN took part in the search for transcription factors. SM provided the study material (part of the snap-frozen samples) and took part in the analysis and interpretation of the obtained data. ND provided background information of certain samples and took part in the analysis and interpretation of the obtained data. VR took part in the analysis and interpretation of the obtained data. JB conceived the study and participated in its design and coordination and helped to draft and revised the manuscript. All authors have read and approved the final manuscript

#### Declaration of competing interests

The authors declare that they have no competing interests.

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## 3.3. Investigations into the suspected correlation of *THADA* and *HMGA2* expression (unpublished results)

After uncovering the negative correlation between the expression of *THADA* and *HMGA2* in dedifferentiated thyroid samples (see section 3.2), the following studies were carried out to test if this association is also true for neoplasias of other tissues in humans. Tested were several groups of hematologic diseases, including acute and chronic, myeloid and lymphocytic leukemia (AML, CML, ALL, CLL), carcinomas of the lung and the prostate, several cell lines, and as non-neoplastic specimens fetal placenta samples. Overall, the correlation could not be confirmed in the above tissues, restricting it at the present state of knowledge to the thyroid (table 2). Although in case of prostate carcinomas with a low Gleason score and in thyroid cell lines results might hint at a tendency (see section 3.3.1. and 3.3.3., respectively), but increasing the number of samples is necessary for final evaluation. This might also be the case for carcinomas of the lung, especially for squamous cell carcinomas, in which initial analysis yielded a highly significant correlation, but further tests challenged these results. All expression data gathered in the studies can be found in the appendix (section 10).

The relatively low to very low variation in expression level of *THADA* in comparison to *HMGA2* resulted in no statistically significant differences between clinical subgroups (defined by differences in type of tumor, histological grading, tumor stage, or patient's age) in any of the investigations.

Overall, in combination with the findings from the study presented in section 3.2, the results further underline the particular role of *THADA* in the thyroid.

**Table 2:** Summary of the statistical analysis of *THADA*- and *HMGA2*-expression in human tissues and blood.

| study | number of samples | $R^2$ | *p* |
|---|---|---|---|
| cell lines | 9 | 0.0542 | 0.5466 |
| carcinomas of the lung | 18* | 0.0421 | 0.4143 |
| prostate carcinomas | 14 | 0.0483 | 0.4503 |
| hematologic diseases | 55 | 0.0595 | 0.0728 |
| fetal placenta | 106 | 0.0310 | 0.0712 |

For each study, numbers always only include samples with data for both genes available. *: In each case, expression data from tumor samples were normalized by the value from corresponding normal tissue specimens from the same patient (see also text, section 3.3.2).

Own contribution:

Study design with Jörn Bullerdiek

Execution and analysis of the study on cell lines, carcinomas of the lung, prostate carcinomas, and hematologic diseases for *THADA* expression except for RNA isolation for part of the samples

Execution and analysis of the study on fetal placenta samples for *THADA* and *HMGA2* expression except for RNA isolation for part of the samples

Statistics with Christian Astrosini

### 3.3.1. Cell lines

In addition to the primary samples (sections 3.3.2. to 3.3.5.) nine cell lines were tested. Among these, five derived from thyroid tumors (two adenomas, two follicular carcinomas, and one anaplastic carcinoma). Also tested were four cell lines derived from other neoplasias, in two cases from T-cell leukemias, one derived from a pleomorphic adenoma of the salivary glands and one from a lipoma.

Relative qRT-PCR data of *HMGA2* was kindly provided by Anke Meyer (Center for Human Genetics, University of Bremen). Part of the *THADA* expression data was published in Drieschner *et al*. (2006). Results from statistical tests clearly indicated no correlation between the expression of *THADA* and *HMGA2* ($R^2$ = 0.0542; $p$ = 0.5466). A positive trend

was suggested when testing the cell lines derived from thyroid tumors alone ($R^2$ = 0.5422; $p$ = 0.1559), whereas the strength of correlation further decreased for the remaining specimens in a joint group ($R^2$ = 0.0195; $p$ = 0.8605).

Six additional cell lines were tested for *THADA* expression. Four of these derived from thyroid adenomas, one of an anaplastic thyroid carcinoma, one was a primary culture of skin fibroblasts. Choosing the cell line with the lowest expression as the calibrator, a maximum fold change of 14.95 times was observed. The Mann-Whitney-U test revealed no significant difference in expression between the cell lines deriving from thyroid tumors and those from non-thyroid specimens.

### 3.3.2. Carcinomas of the lung

Matched normal tissue and tumor samples from 18 patients with carcinomas of the lung were analyzed. All specimens were non-small-cell lung carcinomas, 50 % were adenocarcinomas, the other half squamous cell carcinomas. Five tumors were of grade 1, six were of grade 2, and seven of grade 3. Except for one patient, in all cases stage of the disease was also stated.

Absolute quantification qRT-PCR data of *HMGA2* was kindly provided by Britta Meyer (Center for Human Genetics, University of Bremen). The data for *HMGA2* was published in Meyer *et al*. (2007a). Initial analysis with all 36 samples showed a highly significant correlation between the expression of *THADA* and *HMGA2* ($R^2$ = 0.3135; $p$ = 0.0004). This result could not be reproduced for the group of adenocarcinomas ($R^2$ = 0.0969; $p$ = 0.2085), but for the squamous cell carcinomas, the correlation proved to be even stronger ($R^2$ = 0.6393; $p$ < 0.0001). Further tests showed significant or highly significant correlations in the subgroups of samples from patients with tumor grade 1, grade 2, stage 1 and stage 2. Due to the limited number of samples available, substagings (“A” and “B” suffixes) were neglected. Testing the tumor specimens alone, the results were very similar. Only for the subgroups “grade 2” and “stage 1” the outcome differed, for these $p$ was above 0.05. Regarding the normal samples, only for samples from patients with a squamous cell carcinoma a significant correlation could be detected.

To eliminate a bias potentially due to divergent baseline expression in the lungs of different patients, a normalization of expression values was applied as follows: Each value from a tumor sample was divided by the matched normal tissue's value, i.e. an expression value

relative to the corresponding normal specimen was generated. For these 18 values, the same statistical tests as above were utilized. Comparing the *THADA* and *HMGA2* expression following this method, there was neither a significant difference observed for all samples ($R^2$ = 0.0421; $p$ = 0.4143), nor one for any of the subgroups mentioned above.
Visual assessment of the corresponding scatterplot from the analysis with all 36 samples (not normalized) with *HMGA2* expression values on the x-axis and *THADA* values on the y-axis showed an almost linear distribution of data points along the x-axis, with only four outliers above this virtual line on the right, putting the results from the initial analyses in doubt (data not shown). Additionally, since the corresponding normal tissue of tumor samples cannot have a grading, staging and cannot be regarded as adenocarcinomas or squamous cell carcinomas, and therefore not separated as such, the latter statistical method (normalized values) was used for final evaluation.
Analysis of the expression data of *THADA* showed a range of 1 to 5.17 times. No significant difference in *THADA* expression level could be detected between the subgroups, separating the samples by histological category, age of the patient, stage, or grade. These results were in concordance independent of testing all samples, the tumor or the normal specimens separately, or using the normalized values. There was also no significant difference in *THADA* expression between all normal and all tumor samples, nor in any of the subgroups.

### 3.3.3. Prostate carcinomas

Fourteen prostate cancer samples were investigated. One specimen had a Gleason score of 5, for three samples it was 6, for another three it was 7, four cases had a score of 8, and for yet another three it was 9. In each case, the Gleason grading was also stated. Relative quantification qRT-PCR data of *HMGA2* was kindly provided by Anke Meyer (Center for Human Genetics, University of Bremen). No correlation between the expression of *THADA* and *HMGA2* could be detected ($R^2$ = 0.0483; $p$ = 0.4503). This was also true when testing the samples separated by Gleason grading and by Gleason score, respectively. Although in both cases the coefficient of determination was above 0.9 within the subgroups of lower scores 6 and 7, a $p$-value well above 0.05 indicated no significance, possibly due to the low sample size. Only a single sample had a score of 5, hence no $R^2$ could be determined. The same results were obtained within the subgroups of those gradings resulting in

Gleason scores below eight. For the specimens with a higher score (8 and 9) and corresponding grading, $R^2$ showed a progressive decline, $p$ a further incline.
There was one additional sample available for assessment of *THADA* expression, a tumor with a Gleason score of 6. Overall, *THADA* expression ranged from 1 to 3.31 times. A significant difference in expression could neither be detected when separating the samples by Gleason grading nor by Gleason score.

### 3.3.4. Hematologic diseases

Forty-eight samples of hematologic neoplasias (15 AMLs, five CMLs, seven ALLs, three CLLs, eight other myeloproliferative neoplasms, and ten malignant lymphomas) were investigated, accompanied by seven outside controls from patients with other diseases (two other neoplasias, two cases of systemic lupus erythematosus, and three other non-neoplastic diseases). Seventeen samples were taken before start of the therapy, 26 during and eleven after the end of the therapy, and for one specimen there was no information available about when the sample was taken. In all cases the patient's age, and in all but two the blood cell concentration was stated.
Absolute quantification qRT-PCR data of *HMGA2* was kindly provided by Britta Meyer (Center for Human Genetics, University of Bremen). Five specimens were part of the investigation published in Meyer *et al*. (2007b). Performing a linear regression analysis including the *THADA* and *HMGA2* expression values from all 55 samples, the coefficient of determination amounted to 0.0595 with a $p$-value of 0.0728, suggesting that the expression levels are not correlated. Checking individual disease types alone, $R^2$ was between 0.0223 (ALL) and 0.2632 (malignant lymphoma); the F-test revealed no significant correlation in any of these subgroups. Neither could there one be detected in the joint group of hematologic neoplasias nor in the outside controls taken alone ($R^2$ = 0.0597 and 0.1856, respectively). When grouping the samples by phase of therapy, a $p$-value of 0.0154 ($R^2$ = 0.2210) could be ascertained for the specimens taken during therapy if no outliers were excluded. Nevertheless, further analysis showed that these numbers depended on a single sample, when retesting the subgroup without this specimen (25 samples remaining), the slope of the regression line was reversed and no longer significant ($R^2$ = 0.0685; $p$ = 0.2062). Both subgroups representing samples taken before as well as after therapy showed no significant correlation, either.

In addition to the samples with data for both genes a further eight specimens were analyzed for the expression of *THADA* alone. Seven samples were from healthy subjects, i.e. normal blood samples, one was from a patient with CML. This specimen was taken during therapy. For the normal blood samples, age of each individual and cell number were not available. Overall, for *THADA* the expression range for the specimen with the lowest and the one with the highest value was 1 to 26.31 times. There was no statistically significant difference in expression levels observed between individual disease types, the joint groups of hematologic neoplasias versus joint outside controls versus normal samples, or different therapy phases. Nor could a significant correlation between the expression of *THADA* and the patient's age be detected. When comparing the expression with the cell number, results indicated there was only a very weak correlation between *THADA* expression and the amount of cells per liter blood ($R^2$ = 0.1194; $p$ = 0.0105).

### 3.3.5. Fetal placenta

All samples from section 3.4 (Kloth *et al.*, in preparation) were also tested for *THADA* expression as part of this study. These consisted of 90 fetal placenta specimens from calendar gestational ages (CGA) of five up to 41 weeks. Additionally, eight samples without information about the GA were also investigated. These were accompanied by two hydropic abortions (HA), two partial hydatidiform moles (PHM) and four complete hydatidiform moles (CHM). For these, in five cases the GA was stated. Of the 106 samples, 102 were collected after an early termination of the pregnancy, of these, 70 were spontaneous, 21 were induced abortions (none with medical indication), and for eleven there was no information about the type of abortion. Four specimens were gathered postnatally.

Neither could a correlation between the expression of *THADA* and *HMGA2* in fetal placenta be detected for the non-molar pregnancy samples ($R^2$ = 0.0303; $p$ = 0.0864), nor for the molar pregnancy and hydropic abortion specimens in a joint group ($R^2$ = 0.2533; $p$ = 0.2036). This is also true for all samples combined as well as within each distinct abortion type subgroup. When comparing the expression values for only the samples from the first trimester, $R^2$ (0.1215) and $p$ (0.0019) indicated a significant yet marginal correlation. These numbers were almost identical when omitting the molar pregnancy and hydropic abortion specimens ($R^2$ = 0.1222; $p$ = 0.0026). For the samples from the second

and third trimester combined, no significant correlation was observed ($R^2$ = 0.1975; $p$ = 0.0647).

Considering all samples, *THADA* expression showed a range of 1 to 13.05 times, which was also the case for the non-hydropic placenta samples considered alone. For the hydropic abortions and hydatidiform moles the difference in expression between the sample with the lowest value to the one with the highest one was 1.65 times. The same statistical tests as presented in section 3.4 (Kloth *et al*., in preparation) used to analyze the expression of *HMGA2* were performed to evaluate the one of *THADA*. In contrast to the former, *THADA* expression showed no significant differences between the groups in any regard.

## 3.4. Quantitative analysis of *HMGA2* in fetal placenta

### Publication III: Expression of *HMGA2* in fetal placenta correlates with gestational age (Kloth *et al.*, in preparation)

Following the elucidation of the very low correlation between the expression level of *THADA* and *HMGA2* in fetal placenta samples of the first trimester, the focus was directed towards the study of *HMGA2* in this tissue. While no relation between *THADA* and the CGA could be detected, for *HMGA2* a highly significant correlation was discovered. Samples as described in section 3.3.5. were analyzed, those without information about the CGA were not used for this study. For all specimens the coefficient of determination was 0.413, for those collected after an induced abortion or after birth it turned out to be 0.791, and for the specimens gathered after a spontaneous abortion it was 0.186. In each case, *p* was below 0.001. *HMGA2* expression was high in the early gestation. Around the end of the first trimester the level showed a decline up to a CGA of 28 weeks, after this, only a baseline expression was detectable. Hence, there was a highly significant difference between samples from the first trimester and those from the joint second and third trimester. For the most part, immunoreactivity was consistent with the qRT-PCR. Samples with a low CGA showed intense signals, whereas those from specimens from late stages of pregnancy were barely detectable. Throughout all samples, there was a clear pattern for the localization of HMGA2. Relative to each specimen's level of staining, the strongest signals were detectable in the nuclei of the stroma cells, slightly less intense staining was visible in the cytoplasm of the trophoblast.

## III

## Expression of *HMGA2* in fetal placenta correlates with gestational age

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in preparation

Own contribution:

Study design with Jörn Bullerdiek

Execution and analysis of the study except for immunohistochemistry

Statistics with Werner Wosniok

Writing of the manuscript

# Expression of *HMGA2* in fetal placenta correlates with gestational age

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**Abstract**

**Background**

*High-mobility group AT-hook 2 (HMGA2)* expression can be detected in many embryonic and fetal tissues but becomes down-regulated in adult tissue. The placenta is essential for the development of the embryo and the fetus. It grows rapidly in the first weeks of gestation. There are several research papers discussing genes involved in the development of the placenta. However, this is the first investigation measuring *HMGA2* expression throughout the whole duration of pregnancy.

**Methods**

Expression of *HMGA2* and *HPRT* was measured on 90 fetal placentas, encompassing calendar gestational ages (CGA) of five to 41 weeks, using quantitative real time-PCR (qRT-PCR). In eleven cases, an antibody specific for HMGA2 was used to determine the localization of the protein and to verify the qRT-PCR data.

**Results**

The expression of *HMGA2* is highly significantly correlated with the gestational age ($p < 0.001$). For the better part of the first trimester the level of *HMGA2* is high, after that the expression shows a decline down to a baseline level, where it remains until the birth. HMGA2 protein was mainly detected in the nucleus of the stromal cells in the placental villi.

**Conclusions**

In fetal placenta the expression of *HMGA2* follows a certain pattern. In the first trimester, from two to three weeks after the implantation of the conceptus until the blood supply is established, the expression is high, indicating a critical role in the early development of this organ.

**Background**

The HMGA-(High-mobility group AT-hook-) group is a family of non-histone chromatin proteins, encoded by two genes, *HMGA1* and *HMGA2*. Due to alternative splicing, there are four known proteins (HMGA1a, HMGA1b, HMGA1c, and HMGA2) [reviewed in 1,2]. HMGA2-proteins are architectural transcription factors and contain three DNA-binding domains, called AT-hooks, and an acidic carboxy-terminal tail. As such, they do not have an intrinsic transcription factor capacity, but rather enhance or silence transcription through a change in chromatin structure and interaction with nuclear proteins [reviewed in 3,4]. They play a key role in the mammalian growth and development of tissues and, in some cases, the differentiation of cells [5-9]. Especially in the case of *HMGA2*, the expression is not detectable in most adult tissues with the exception of spermatocytes and spermatides in testis [10-14]. *HMGA* reactivation in adult tissue was first reported in HeLa cells [15]. Since then, the overexpression of *HMGA1* and -*2* was detected in numerous types of cancer cells [reviewed in 1,2,16].

The placenta is the only normal human tissue which shows infiltrating behavior, albeit, as a rule, in a tightly restricted manner. It serves as the connection between the mother and the embryo or fetus, providing nutrients and oxygen for the developing child. Additionally, it removes waste products from the fetus and forms a maternal-fetal barrier, protecting the fetus from microbes and cloaking it from the mother's immune system. About six days after fertilization, the blastocyst begins the implantation into the maternal uterus and starts the placentation. Five weeks after conception the basic structure of the placenta has formed. To support the developing fetus, it continues to grow throughout gestation.

The expression of HMG-proteins in the placenta has been reported as early as 1987. Corfman *et al*. [17] discovered the presence of HMGB and -N in human term placenta. The detection of *HMGA2* expression in the placenta dates back to 1996 [11]. To detect *HMGA2* mRNA, RT-PCR was used on several fetal tissues of a gestational age between eight to twelve weeks and on maternal and fetal placenta of 36 weeks. The experiments yielded positive results for the embryo, but negative findings for the placenta. In another investigation [13] RNA in situ hybridization was used on mouse embryo sections for detection of *HMGA2* mRNA, including fetal placenta. Here - in comparison to nearly all parts of the embryo - reduced expression of *HMGA2* was found at a developmental stage of 9.5 [13]. *HMGA2* mRNA was also detected by RT-PCR, but not by northern blot analysis

in three fetuses of 19 to 22 weeks estimated gestational age by Gattas *et al*. [18]. Genbacev *et al*. [19] identified the chorionic mesoderm as a niche for human trophoblastic progenitor cells that support placental growth. *HMGA2* was found to be one of the factors associated with the self-renewal or differentiation of these cells.

The aim of the present paper was to determine if there is a temporally and spatial expression pattern of *HMGA2* in the fetal placenta. In case of a correlation between the pattern and the invasive growth of the placenta, this would provide first insights into the role of *HMGA2* in the development of this organ.

## Methods

### Tissue specimens collection and RNA isolation

Formalin-fixed paraffin-embedded (FFPE) tissue samples were collected at the Institute for Pathology, Elbe Clinic Stade-Buxtehude, Germany. Pathological examinations were performed after haematoxylin and eosin staining of the samples. Depending on the size of the embedded tissue, FFPE blocks were cut into six to eight sections of 5 µm for each sample using a microtome. Total RNA isolations were performed using the innuPREP Micro RNA Kit (Analytik Jena AG, Jena, Germany) for RNA isolation according to the manufacturer's instructions with the following modifications: Lysis of the paraffin sections preceding RNA isolation was conducted using TLS-Lysis Solution and Proteinase K from the innuPREP DNA Micro Kit (Analytik Jena AG) without prior deparaffinization. Sections were incubated for 1 h at 60°C and 15 min at 80°C.

### cDNA-synthesis and quantitative real-time RT-PCR

RNAs were reverse-transcribed into cDNA by M-MLV Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). Real-time PCR was performed using the Applied Biosystems 7300 sequence detection system according to Taq-Man Gene Expression Assay Protocol (Applied Biosystems, Darmstadt, Germany) in 96-well microtiter plates with a total volume of 20 µl. In the case of the TaqMan gene expression assay for *HMGA2* (assay number Hs00171569, Applied Biosystems, Foster City, USA), each reaction consisted of 2 µl of cDNA reverse transcribed from 25 ng of total RNA, 10 µl of TaqMan Universal PCR Master Mix (Applied Biosystems), 1 µl of TaqMan assay and 7 µl of $ddH_2O$. For the *HPRT* assay,

using HPRT FP and HPRT RP primers [20], each reaction consisted of 2 µl of cDNA reverse transcribed from 25 ng of total RNA, 10 µl of TaqMan Universal PCR Master Mix, 600 nM (1.2 µl) of forward and reverse primers, 200 nM (0.2 µl) of probe [20] and 5.4 µl of $ddH_2O$. Thermal cycling conditions were 2 min at 50°C followed by 10 min at 95°C, 50 cycles at 95°C for 15 s and 60°C for 1 min. For each sample, a negative control of a previous cDNA synthesis (missing reverse transcriptase) and for each plate a non-template control of amplification and a non-template control of previous cDNA synthesis were included in each run. Sequence Detection Software 1.2.3 (Applied Biosystems) was programmed with the reaction conditions. All testing reactions were performed in triplicate. Considering the expression range of *HMGA2*, *HPRT* was used as endogenous control, as it has previously been shown to be stably expressed in human fetal placenta [21-23]. As recommended for FFPE samples [24] the fragment sizes amplified by both assays were small, ranging between 65 and 80 bp. A validation of these values was performed via gel electrophoresis of the PCR (data not shown). When applying the comparative $C_T$ method, the sample with the lowest value was used as calibrator.

**HMGA2 immunohistochemical analysis**

Slides utilized for the immunohistochemical analysis were produced using cuts directly adjacent to those employed for the qRT-PCR investigation. Immunohistochemical staining for HMGA2 (rabbit polyclonal anti-HMGA2-P3, Biocheck, Inc., Forster City, USA) was performed using a detection kit (DAKO ChemMate; DAKO, Glostrup, Denmark) and a semi-automated stainer (DAKO; TechMate) according to the specifications of the manufacturer. For antigen retrieval the slides were treated in a PT Link module (DAKO) using the EnVision™ FLEX Target Retrieval Solution, low pH (DAKO). The antibody dilution used was 1:500.

**Statistical analysis**

The two-sided Wilcoxon rank sum test was used to compare averages from two independent groups. Relationships between two observed or measured amounts were quantified by linear regression or by a nonparametric spline model, if the latter turned out to have a significantly better fit. Models were compared by the likelihood ratio test. A *p*-value of less than 0.05 was considered significant, a *p*-value below 0.001 was deemed

highly significant. Statistical calculations were done using the R package, version 2.15 [25].

**Ethics Statement**

All samples investigated were initially taken for diagnostic purposes and secondarily used for the present study. Samples were deidentified before their use in this study, in line with the rules of the Helsinki declaration. The study was approved by the local ethics committee (Ärztekammer Bremen, reference number 371).

**Results**

**qRT-PCR analysis**

Ninety samples of human fetal placenta were tested for the expression of *High-mobility group protein AT-hook 2* (*HMGA2*) mRNA (table 1A and 1B). Eighty-six were collected after an early termination of the pregnancy. Of these abortions, 64 were spontaneous, 19 were induced (none with medical indication). In three cases, no information was available about the type of abortion. In addition, four specimens collected after birth were examined.

The placenta samples showed a relative expression ranging from 1 to 498 (fig. 1). Overall, there was a strong correlation between gestational age and the level of *HMGA2* expression. For all placenta samples, the correlation coefficient was 0.6425 ($p$ = 8,73*10$^{-12}$) (fig. 2). When the analysis was constrained to the specimens gathered after induced abortion (IA) and after delivery (AD), the $r$-value was 0.8894 ($p$ = 1,62*10$^{-7}$) (fig. 3). The samples collected after a spontaneous abortion (SA) showed a correlation coefficient of 0.4312 ($p$ = 3.75*10$^{-4}$) (fig. 4). The relation between gestational age and *HMGA2* expression was found not to be significantly different between these groups ($p$ = 0.079, likelihood ratio test).

*HMGA2* levels from samples taken during the first trimester of pregnancy differed from those of the joint second and third trimester highly significantly ($p$ = 2.29*10$^{-6}$, Wilcoxon signed rank test). Using the same test, significant differences were also found within the SA subgroup ($p$ = 0.00938) and within the IA and AD joint subgroups ($p$ = 1.98 * 10$^{-5}$).

Taking all samples into consideration, a relatively wide ranging but overall high level of expression was observed up to the calendar gestational age (CGA) of 13 weeks, which

marks the end of the first trimester. After that, the level of expression showed a decline up to the CGA of 28 weeks and finally leveled out to a steady value for the rest of the pregnancy. In non-spontaneous abortion specimens, the observed drop was more pronounced and happened earlier, at around nine weeks of gestation.

Pathological examinations of the specimens after haematoxylin and eosin staining revealed a considerable contamination of maternal decidua in several samples (see also table 1). After the immunohistochemical analysis (see also below), the decidua was found to be HMGA2 negative (data not shown). Therefore, it seems conceivable that the presence of this tissue is the reason for low *HMGA2* expression in some of the early gestation samples. In two specimens, part of the umbilical cord was detected. Human umbilical cord blood-derived stromal cells (hUCBSCs) show high *HMGA2* expression in culture [26,27]. Even though no data is available for fresh tissue samples, this might in part explain the outliers with a relative high expression in later stages of the pregnancy.

**Immunohistological analysis**

Eleven samples were investigated for the presence and localization of HMGA2 via immunohistochemical analysis. Overall, the results from the immunostaining are in concordance with those from the qRT-PCR, except for two relatively mild outliers. Samples from the early gestation (eight to ten weeks CGA) showed intense signals (fig. 5A). The strongest staining was visible in the nuclei in the stroma cells, slightly less intense signals were detectable in the cytoplasm of the trophoblast. Samples from gestational age between 19 and 41 weeks showed only weak to very weak signals (fig. 5B). For case number 34 (eight weeks CGA) the qRT-PCR suggested a relatively low expression in comparison to other samples of the same developmental stage, but the signals for the protein were strong (fig. 5C). For case no. 52, the measured mRNA level was above the average value for the developmental stage, but after the immunostaining only a weak signal was visible on the slide (fig. 5D).

**Discussion**

To quantify the amount of *HMGA2* mRNA in human fetal placentas throughout the pregnancy, 90 samples encompassing calendar gestational ages (CGA) from five to 41

weeks were investigated using quantitative real-time PCR. A highly significant correlation was found between the level of expression of *HMGA2* and the CGA. The level of *HMGA2* is high in the early CGA samples and declines towards the end of the first trimester, followed by a stable level until birth. In the samples from induced abortions and placentas collected after birth, the *HMGA2* level drops around the ninth week of CGA. Considering all samples, the decrease is less steep and takes place several weeks later. For the specimens collected after a spontaneous abortion, it is conceivable that the underlying cause of the abortion affected the development of the placenta and the expression of *HMGA2*. In addition, the CGA is less precise, because the embryo or fetus might have died days or even weeks before abiosis of the placenta [28]. Therefore, despite a smaller sample size, more reliable results may be obtained from the group of specimens gathered after induced abortions and after birth. Differences in *HMGA2* expression between the samples at an identical specified CGA might be explained by the uncertainty of the exact age of the embryo or fetus. In a group of 29 pregnant women, Blaas *et al*. [29] found virtually the same growth velocities for the embryo or fetus, respectively, between seven and twelve weeks of gestation, but considerable differences between the individuals. As a reason, the authors discuss discrepancies between the CGA and the true gestational age and differences in development before week seven of the pregnancy as possible explanations [29]. Following this argumentation and transferring it to the development of the fetal placenta, differences in *HMGA2* expression might also be related to the uniqueness of each placenta's development in the very early course (before week nine CGA) of the pregnancy.

The stable level of *HMGA2*-expression detected in samples from the third trimester contrasts with the analysis by Rogalla *et al.* [11], where no *HMGA2*-expression was found in fetal term placenta. A possible explanation is the higher sensitivity of qRT-PCR compared to conventional RT-PCR.

*HMGA2* is known to be participating in the proliferation of tissues by upregulation of genes that are involved in cell proliferation and invasion [reviewed in 2,30]. Therefore, results presented in this study do not contradict those published by Sitras *et al.* [31], who tested more than 29,000 genes, including several members of the *HMG*-family, but not *HMGA2.* Applying microarray analysis, the authors found that genes involved in cell proliferation, differentiation, and angiogenesis were upregulated in human placenta in the first trimester

[31].

High *HMGA2* expression coincides with the uterus being a low oxygen environment. As a result of trophoblast invasion into the maternal decidua, spinal arteries are plugged throughout the first trimester [reviewed in 32]. Filtrated plasma enriched with secretions from the endometrial glands can be found in the placental intervillous space, providing histiotrophic nutrition [33]. A low oxygen environment has been shown to be necessary for the proliferation of cytotrophoblast cells [34,35]. Around week 7 to 8 of gestation (week 9 to 10 CGA), maternal uterine circulation to the placenta begins [36, reviewed in 37], resulting from trophoblast (extravillous) invasion of capillaries. This marks the beginning of the haemotrophic nutrition of the fetus, which coincides with the decrease of *HMGA2* expression.

Scheduled proliferation and invasion of trophoblast cells are crucial for an undisturbed pregnancy. Superficial implantation of the placenta leading to poor placental and uterine perfusion is characteristic for preeclampsia [38,39]. No known cure exists for this serious medical condition with a maternal mortality rate of 20 % to 80 % [40], other than delivery of the fetus and placenta. Preeclampsia is not yet fully understood, and there are few markers for diagnosis [41]. In combination with oxidative stress of the placenta [reviewed in 28], several transcription factors involved in the proliferation and differentiation of the trophoblast have been detected to have a share in an elevated risk of preeclampsia [42-45]. For some proteins, a significantly higher expression has been shown in early gestation [43,44,46], similar to the results for *HMGA2*. While symptoms of preeclampsia do not appear before the 20th week of gestation, it seems to result from earlier changes of proliferation and differentiation of the trophoblast that play a key role in the implantation of the embryo during low placental oxygenation in the first trimester [reviewed in 47]. In this investigation, case number 90 was diagnosed as a severe form of preeclampsia, which led to a premature delivery at the gestational age of 32 weeks (see also table 1). As expected, the measured value for *HMGA2* was distinctly within range for this late stage of pregnancy. In case of a correlation between *HMGA2* and preeclampsia, a deviation already would have been detectable in the first trimester, since, as discussed above, in that period of time the proliferation and invasion of the trophoblast determines whether an elevated risk for preeclampsia will exist. A similar situation was reported by Jeon *et al*. [41] for IMUP-2. The authors suggest an association of this protein with preeclampsia, but their findings show

no difference in expression between term patients with or without preeclampsia.

Whereas shallow infiltration of the trophoblast is a sign for preeclampsia, overly deep infiltration indicates another obstetric complication: placenta accreta (including the closely related forms of increta and percreta). This severe complication during pregnancy has been associated with decidual deficiency and an overinvasive trophoblast [reviewed in 48]. Placenta accreta accounts for about 1 % of maternal mortality in the USA [49], and approximately 5 % of the women with this complication die [50,51]. In addition, fetal deaths occur in almost 26 % of the cases [51]. Since the 1970s, the incidence has risen from one in approximately 4,000 deliveries to one in 333 [50,52-54]. The reasons are not fully understood, making further investigations necessary. However, if a relation with deviant *HMGA2* expression exists, expression measured after a chorionic villus sampling might serve as a diagnostic marker. Since there is no definitive method [55] to detect this complication before birth, a possible test would be of high interest.

During the immunohistochemical analysis it became apparent that *HMGA2* is strongly expressed in the stroma cells of the placental villi. As expected for a transcriptional factor, it was located in the nucleus. But, in a lesser concentration, a signal was also detected in the cytoplasm of the trophoblast. This pattern is significantly different from that of HMGA1, as reported by Bamberger *et al*. [56]. HMGA1 was found exclusively in the trophoblast cells [56]. This suggests different roles for the two members of the HMGA family. The results from this study are in concordance with those from Genbacev *et al*. [19], who found the same distribution of HMGA2 in the villi, as well as a change from nuclear to cytoplasmatic location. Since experiments were conducted in vitro in the study by Genbacev *et al*. [19], the cell culture environment may have influenced *HMGA2* expression in their study. HMGA proteins are considered nuclear proteins [2], even though cytoplasmatic expression of HMGA1 has been reported before [56,57]. In the majority of papers, authors only evaluated nuclear immunoreactivity for HMGA2 [58,59]. An exception is the study by Ding *et al*. [60]. The authors detected cytoplasmatic *HMGA2* expression in bladder cancer cells [60]. A true signal in the cytoplasm of the trophoblast might be conceivable due to the following: Firstly, the distribution of the immunohistochemical signals in the trophoblast was consistent in all samples. Secondly, the relation between the levels of staining of the stroma to the trophoblast, which was identical throughout all specimens. Thirdly, the manufacturer’s statement regarding the specificity of the antibody.

And finally, the aforementioned study by Genbacev *et al*. [19]. As evaluated by the visual assessment of the staining in relation to the developmental stage of the samples, the signal intensity decreased considerably with the age of the placenta, further emphasizing the correlation between the expression of *HMGA2* and the gestational age.

## Conclusions

*HMGA2* expression levels have been found to be high in the early fetal placenta, with a rapid decline around the end of the first trimester. Thus, *HMGA2* may perform essential functions in the early development of the fetal placenta. Overall, these findings could serve as the basis for further studies examining the role of *HMGA2* in gestation. Future research might focus on gathering information on whether a correlation of *HMGA2* expression and placenta accreta and/or preeclampsia or other placental diseases exists.


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## Authors' contributions

LK conceived and designed the study, carried out the molecular genetic studies, took part in the immunohistochemical and the statistical analysis and wrote the manuscript. BMH provided the study material, carried out the pathological determination of the samples, the immunohistochemical analysis, and the clinical workout. WW carried out the statistical analysis. AG took part in the pathological determination of the samples. JB conceived the study and participated in its design and coordination, and helped to draft, and also revised the manuscript. All authors have read and approved the final manuscript.

## Declaration of competing interests

The authors declare that they have no competing interests.

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## Tables

Table 1 - Summarization of all samples investigated for the expression of *HMGA2*.

1A: early termination samples

RQ: relative quantification; CGA: calendar gestational age in weeks; n.a.: information not available; percentage of decidua: sample consisted of at least 50 % decidua; umbilical cord: sample contained a section of the umbilical cord; immunostaining: sample was used for immunostaining with an HMGA2-specific antibody.

1B: samples collected after birth

RQ: relative quantification; CGA: calendar gestational age in weeks; age: age of the mother at delivery; sex: sex of the neonate; weight: weight of the neonate in grams; C section: Caesarean section.

Table 1A:

| case no. | RQ | CGA | type of abortion | note |
|---|---|---|---|---|
| 1 | 140.523 | 7 | spontaneous | |
| 2 | 9.902 | 7 | spontaneous | percentage of decidua |
| 3 | 3.120 | 15 | induced | |
| 4 | 95.469 | 9 | spontaneous | |
| 5 | 46.382 | 10 | spontaneous | |
| 6 | 68.581 | 8 | spontaneous | |
| 7 | 36.058 | 8 | spontaneous | |
| 8 | 32.201 | 8 | induced | |
| 9 | 29.015 | 8 | spontaneous | |
| 10 | 497.846 | 8 | spontaneous | immunostaining |
| 11 | 19.692 | 10 | spontaneous | |
| 12 | 89.261 | 9 | spontaneous | |
| 13 | 67.173 | 6 | induced | |
| 14 | 99.902 | 9 | spontaneous | |
| 15 | 19.433 | 14 | spontaneous | |
| 16 | 45.969 | 10 | spontaneous | |

| | | | | |
|---|---|---|---|---|
| 17 | 49.632 | 7 | n.a. | |
| 18 | 141.657 | 10 | spontaneous | |
| 19 | 4.725 | 11 | spontaneous | percentage of decidua |
| 20 | 13.690 | 9 | spontaneous | percentage of decidua |
| 21 | 3.507 | 28 | spontaneous | |
| 22 | 130.567 | 7 | spontaneous | |
| 23 | 109.029 | 8 | induced | immunostaining |
| 24 | 188.477 | 7 | induced | immunostaining |
| 25 | 9.020 | 10 | spontaneous | |
| 26 | 85.272 | 11 | spontaneous | |
| 27 | 55.483 | 8 | spontaneous | |
| 28 | 136.494 | 7 | induced | |
| 29 | 32.358 | 8 | induced | |
| 30 | 96.846 | 8 | spontaneous | |
| 31 | 64.117 | 7 | spontaneous | |
| 32 | 1.027 | 18 | spontaneous | |
| 33 | 23.595 | 22 | spontaneous | umbilical cord |
| 34 | 68.913 | 8 | induced | immunostaining |
| 35 | 244.977 | 8 | spontaneous | immunostaining |
| 36 | 23.640 | 10 | spontaneous | |
| 37 | 39.999 | 11 | spontaneous | |
| 38 | 24.045 | 9 | spontaneous | |
| 39 | 2.220 | 20 | induced | |
| 40 | 1.057 | 32 | spontaneous | immunostaining |
| 41 | 198.117 | 9 | spontaneous | immunostaining |
| 42 | 87.735 | 7 | induced | immunostaining |
| 43 | 26.091 | 8 | spontaneous | |
| 44 | 8.117 | 9 | spontaneous | |
| 45 | 54.529 | 9 | spontaneous | |
| 46 | 30.706 | 9 | spontaneous | |
| 47 | 9.355 | 7 | spontaneous | percentage of decidua |
| 48 | 80.126 | 8 | spontaneous | |
| 49 | 21.238 | 10 | spontaneous | |
| 50 | 44.867 | 9 | spontaneous | |
| 51 | 1.000 | 7 | spontaneous | percentage of decidua |
| 52 | 152.952 | 19 | spontaneous | immunostaining |
| 53 | 63.482 | 7 | spontaneous | |
| 54 | 250.892 | 11 | spontaneous | |
| 55 | 15.773 | 7 | induced | percentage of decidua |

| | | | | |
|---|---|---|---|---|
| 56 | 160.201 | 7 | spontaneous | |
| 57 | 247.132 | 11 | spontaneous | |
| 58 | 92.824 | 7 | spontaneous | |
| 59 | 74.893 | 9 | spontaneous | |
| 60 | 162.155 | 7 | spontaneous | |
| 61 | 126.681 | 6 | induced | |
| 62 | 186.271 | 9 | spontaneous | |
| 63 | 28.572 | 8 | induced | |
| 64 | 47.861 | 7 | induced | |
| 65 | 31.963 | 9 | spontaneous | |
| 66 | 154.653 | 7 | spontaneous | |
| 67 | 95.824 | 7 | induced | |
| 68 | 31.415 | 9 | induced | |
| 69 | 72.805 | 7 | spontaneous | |
| 70 | 56.650 | 10 | spontaneous | |
| 71 | 110.908 | 20 | spontaneous | umbilical cord |
| 72 | 6.058 | 7 | spontaneous | percentage of decidua |
| 73 | 22.586 | 14 | spontaneous | |
| 74 | 123.656 | 7 | induced | |
| 75 | 28.452 | 9 | spontaneous | |
| 76 | 18.370 | 5 | induced | percentage of decidua |
| 77 | 20.153 | 7 | induced | percentage of decidua |
| 78 | 87.926 | 8 | spontaneous | |
| 79 | 109.971 | 12 | spontaneous | |
| 80 | 152.111 | 5 | spontaneous | |
| 81 | 128.672 | 7 | spontaneous | |
| 82 | 47.139 | 9 | spontaneous | |
| 83 | 3.306 | 28 | n.a. | |
| 84 | 4.502 | 30 | n.a. | |
| 85 | 23.446 | 27 | spontaneous | |
| 86 | 6.276 | 24 | spontaneous | |

1B:

| **case no.** | **RQ** | **GA** | **age** | **sex** | **weight** | **C section** | **note** |
|---|---|---|---|---|---|---|---|
| 87 | 1.557 | 38 | 30 | m | 3155 | no | |
| 88 | 1.711 | 41 | 30 | m | 4250 | yes | immunostaining |
| 89 | 1.525 | 38 | 41 | m | 3435 | yes | immunostaining |
| 90 | 1.698 | 32 | 28 | m | 1750 | yes | |

**Figures**

Figure 1 - *HMGA2* expression in relation to the gestational age.
Linear display for *HMGA2* expression, all samples. Coloring of the rhombi denotes the type of sample, blue: spontaneous abortion, red: induced abortion, green: gathered postpartum, orange: abortion, no information available on the type of abortion.

Figure 2 - *HMGA2* expression in relation to the gestational age including the linear regression line with 95% confidence range.
Logarithmic display for *HMGA2* expression, all samples. Coloring of the rhombi denotes the type of sample, blue: spontaneous abortion, red: induced abortion, green: gathered after delivery, orange: abortion, no information available on the type of abortion.

Figure 3 - *HMGA2* expression in relation to the gestational age including the spline fit with 95% confidence range.
Logarithmic display for *HMGA2* expression, samples collected after induced abortion and after birth. Coloring of the rhombi denotes the type of sample, red: induced abortion, green: gathered after delivery.

Figure 4 - *HMGA2* expression in relation to the gestational age including the linear regression line with 95% confidence range.
Logarithmic display for *HMGA2* expression, spontaneous abortion samples.

Figure 5 - Immunoreactivity for HMGA2.
As mentioned in the text, in all but two cases the qRT-PCR data was in concordance with the interpretation of the HMGA staining. For the outliers, see (C) and (D). (A) case number 24 (seven weeks CGA) shows an intensive signal for HMGA2 and a high expression as measured in the qRT-PCR. (B) in case no. 89 (38 weeks calendar gestational age) the signal is barely visible, the qRT-PCR showed a very low expression. (C) case number 34 (eight weeks calendar gestational age) shows a strong signal, the measured expression was average, (D) in case no. 52 (19 weeks calendar gestational age) the signal is weak, the qRT-PCR data suggested a relatively high expression (see also table 1A and 1B). In all samples with a level high enough for visual detection, HMGA2 was found in the nuclei of the stroma cells of the villi and in the cytoplasm of the trophoblast.

Figure 1:

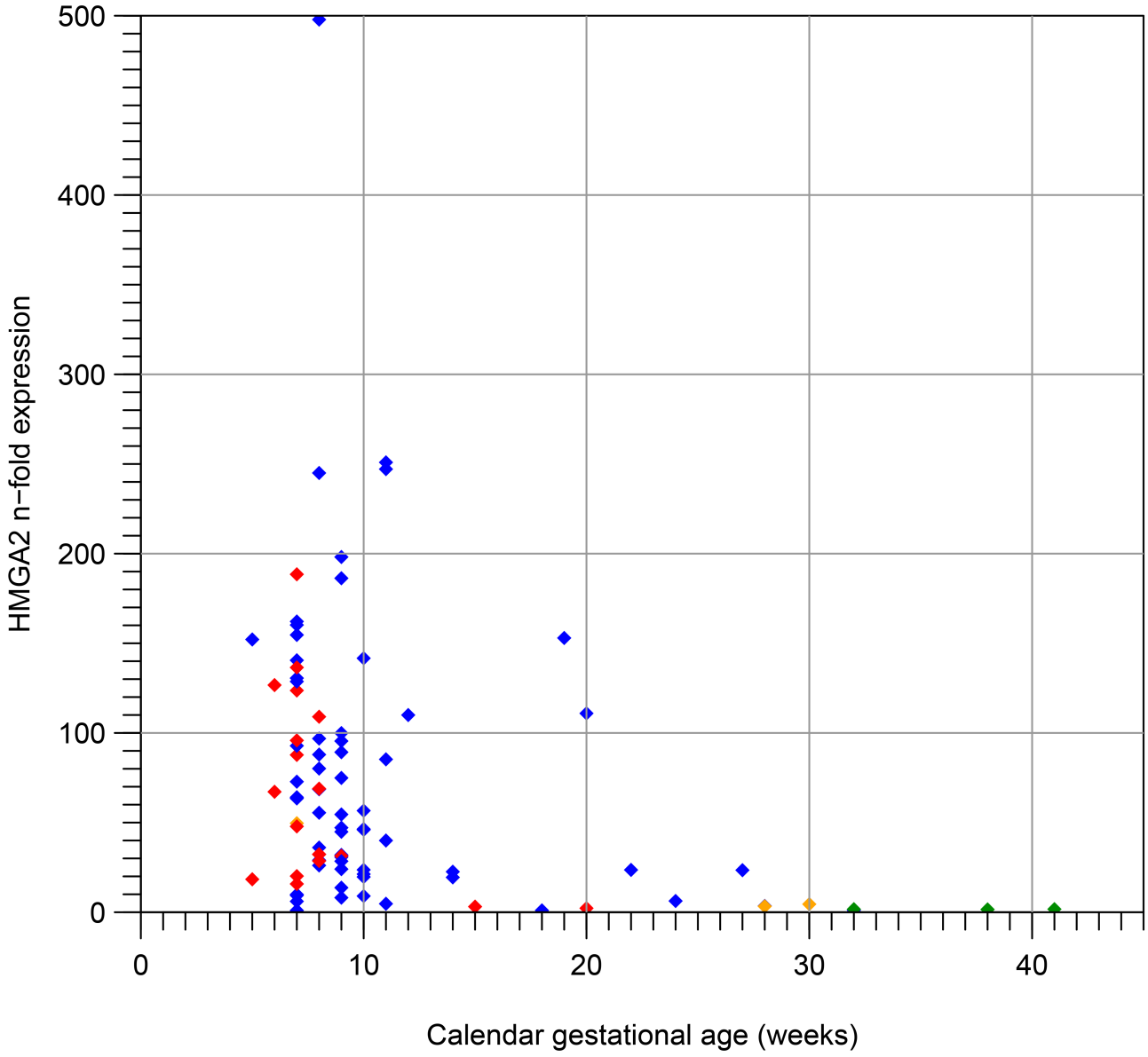
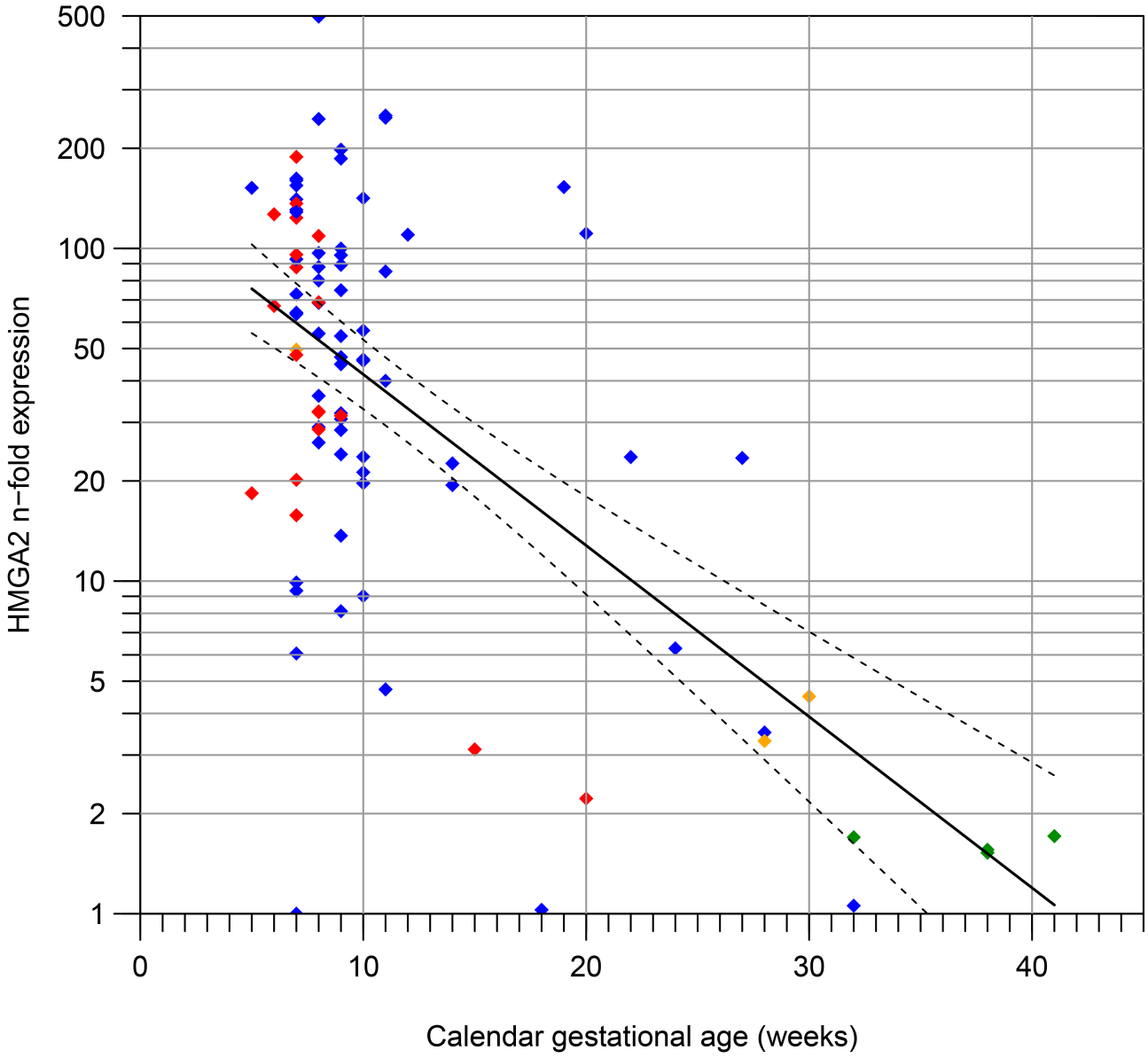

HMGA2 n−fold expression
500
400
300
200
100
0
0
10
20
30
40
Calendar gestational age (weeks)

Figure 2: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HMGA2 n−fold expression
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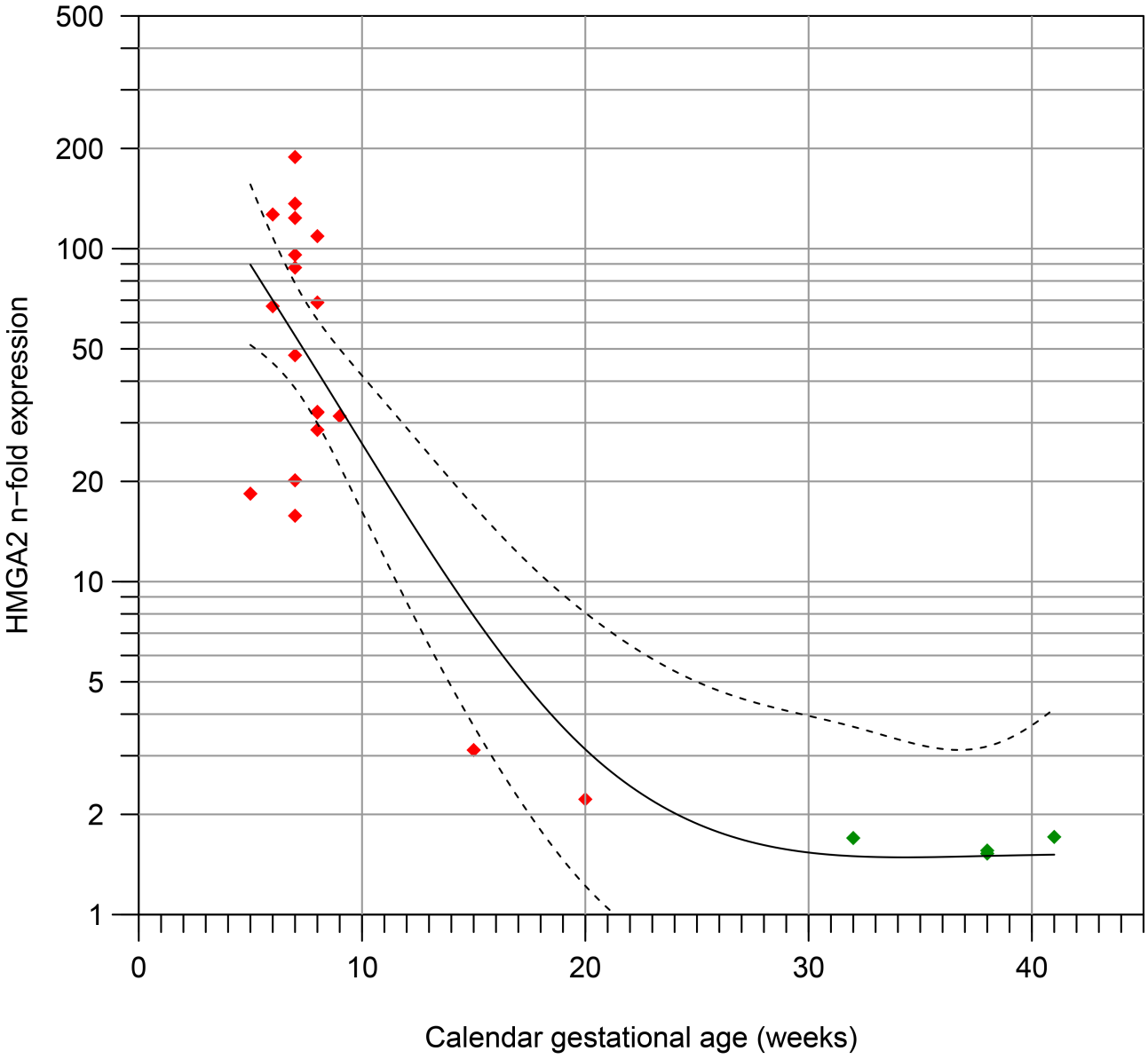
Calendar gestational age (weeks)

Figure 3: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2
1
HMGA2 n−fold expression
0
10
20
30
40
Calendar gestational age (weeks)

Figure 4:

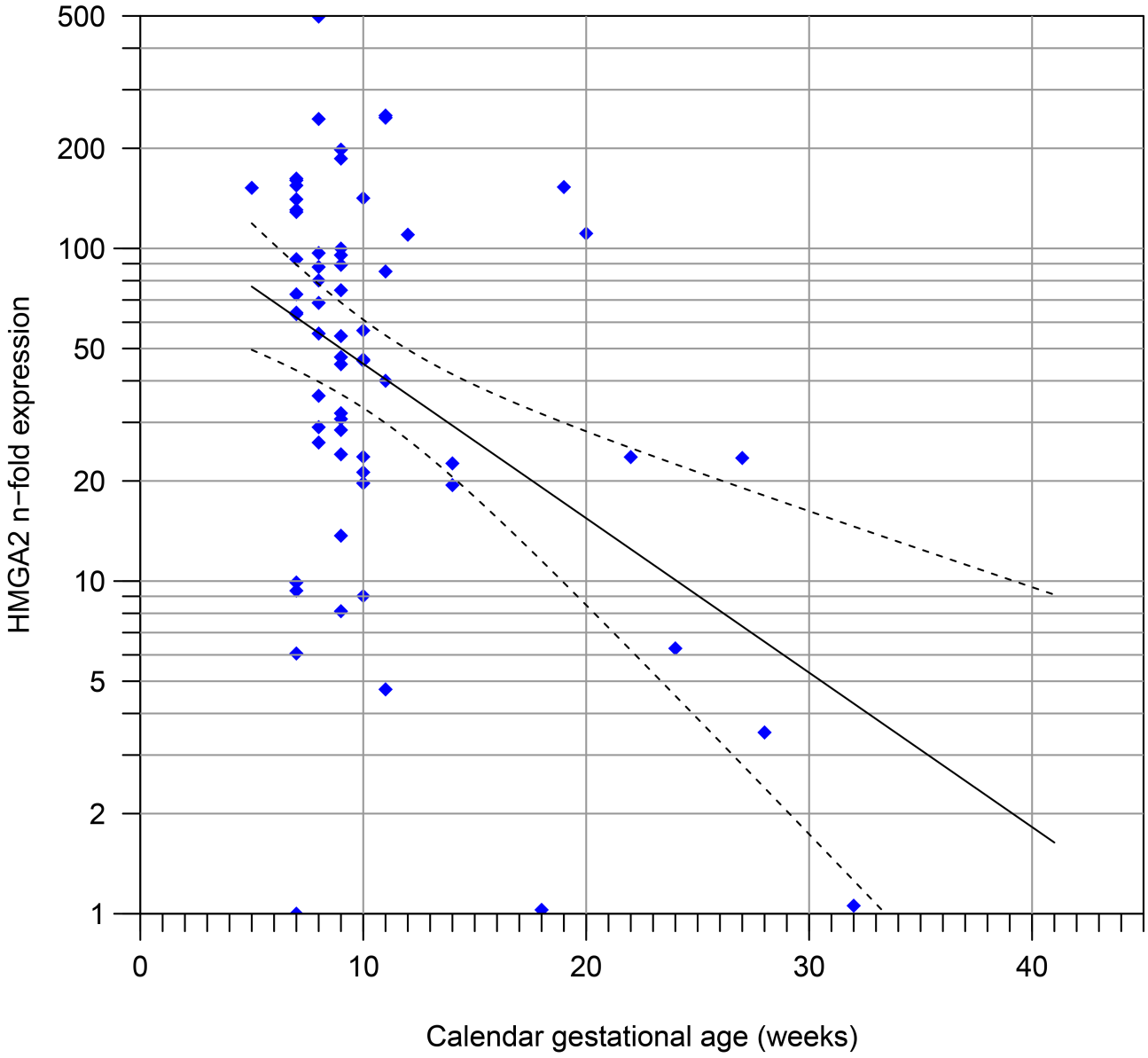
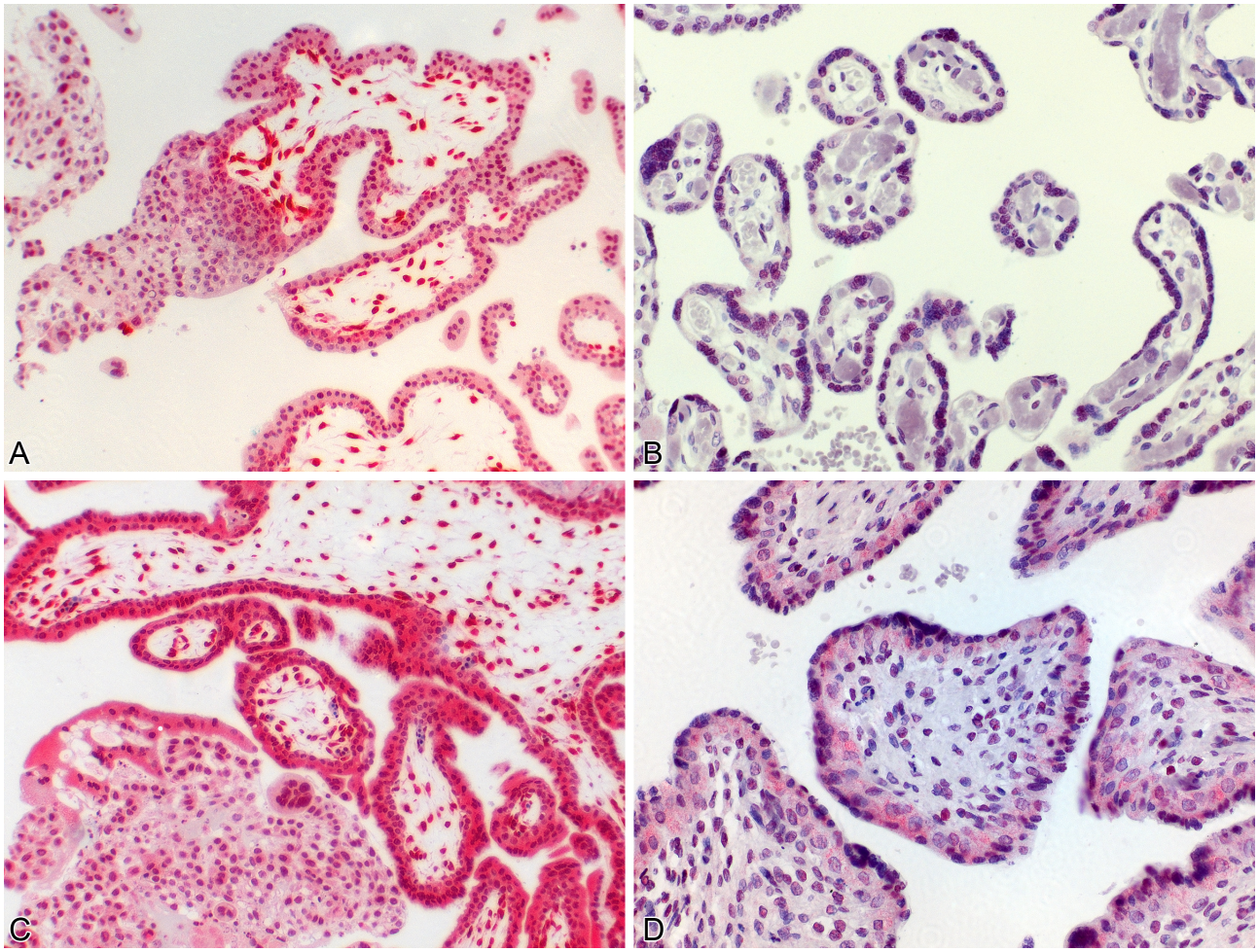

500
200
100
50
20
10
5
2
1
HMGA2 n−fold expression
0
10
20
30
40
Calendar gestational age (weeks)

Figure 5:



## 3.5. *HMGA2* expression in hydatidiform moles (unpublished results)

In addition to the normal placenta tissue samples (see chapter 3.4.), eight specimens, initially determined as hydatidiform moles, were also tested for the expression of *HMGA2*. These samples are identical to those presented in chapter 3.3.5.

The same methods as in the study above were applied with the following exceptions: All samples were investigated for $p57^{KIP2}$ expression via immunoblot and analyzed by fluorescence in situ hybridization (FISH). In six cases an antibody specific for HMGA2 was used to determine the localization of the protein and for verification of the qRT-PCR data.

After assessment of the results, two of the samples initially diagnosed as partial hydatidiform moles were determined as hydropic abortions, one turned out to be a relatively rare PHM with paternal triploidy (table 3). These and the hydatidiform moles showed a very similar pattern of *HMGA2* expression, spatially as well as concerning the level of mRNA and protein, respectively. Quantitative real-time PCR data as well as results from the immunostaining were very similar to the non-molar and non-hydropic placenta samples, with overall a slightly lower level of HMGA2 (mRNA and protein), subsequently there were no statistically significant differences.

**Table 3:** Analysis of hydatidiform moles and hydropic abortions.

| case no. | RQ | GA | initial pathological diagnosis | FISH analysis | $p57^{KIP2}$ immuno-staining | reviewed pathological diagnosis |
|---|---|---|---|---|---|---|
| 1 | 21.294 | n.a. | PHM | triploidy | positive | PHM |
| 2 | 38.615 | 9 | PHM | tetraploidy | positive | PHM w/ paternal triploidy |
| 3 | 112.24 | 7 | PHM | diploidy | positive | HA |
| 4 | 3.872 | 9 | PHM | diploidy | positive | HA |
| 5 | 24.395 | n.a. | CHM | diploidy | negative | CHM |
| 6 | 34.784 | 9 | CHM | diploidy | negative | CHM |
| 7 | 62.57 | n.a. | CHM | diploidy | negative | CHM |
| 8 | 35.189 | 6 | CHM | diploidy | negative | CHM |

RQ: relative quantification, *HMGA2* expression; GA: calendar gestational age in weeks; PHM: partial hydatidiform mole; CHM: complete hydatidiform mole; HA: hydropic abortion.

Own contribution:

Study design with Jörn Bullerdiek

Execution and analysis of the study except for FISH analysis and immunostaining

Statistics with Werner Wosniok

### 3.5.1. Pathological determination

Eight samples of suspected molar pregnancies were investigated (table 3). The initial pathological report stated four cases as complete hydatidiform moles (CHM) and four as partial moles (PHM). After molecular analysis, using (FISH) and immunostaining with an antibody specific for $p57^{KIP2}$, only two samples were still declared as a PHM, the other two were classified as hydropic abortions (HA), the four CHMs were confirmed (table 3 and figures 1 and 2).

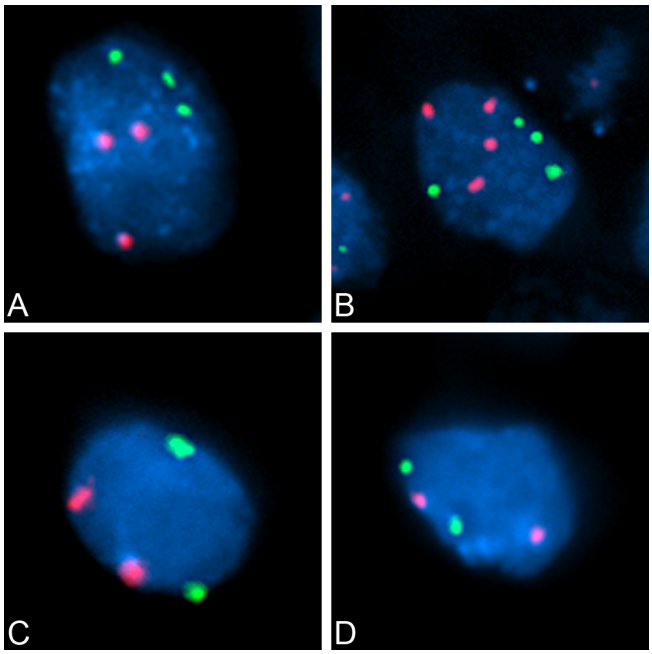



**Figure 1:** FISH analysis of the hydatidiform moles and hydropic abortions.

Representative images of the four groups determined by fluorescence in situ hybridization analysis: hydropic abortions (HA), complete hydatidiform moles (CHM), and partial hydatidiform moles (PHM) using probes specific for chromosomes 1 and 6, respectively. (A): triploid PHM (case number 1, see also table 2); (B): tetraploid PHM (case number 2); (C): diploid HA (case number 4); (D): diploid CHM (case number 8).

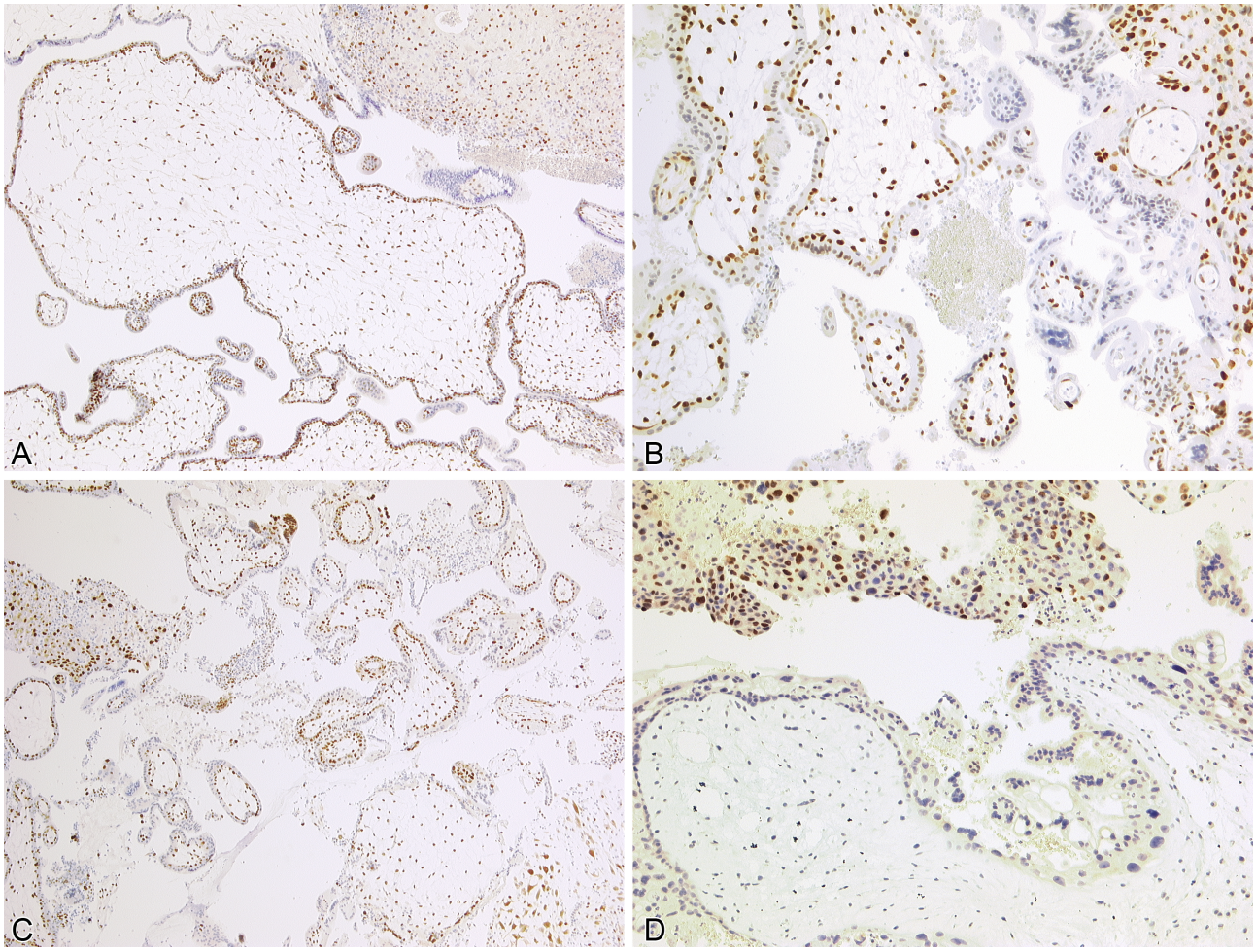



**Figure 2:** Immunoreactivity for $p57^{KIP2}$ in HA, PHM, and CHM samples.

Triploid PHM (A), tetraploid PHM (B), and HA (C) samples show nuclear immunoreactivity for $p57^{KIP2}$ in stroma and cytotrophoblast cells, in the CHM sample (D) only the maternal decidua is positive for the staining. Pictures (A) to (D) correspond to case numbers 1, 2, 4, and 6, respectively, in the investigation (see also table 2).

### 3.5.2. qRT-PCR analysis

Overall, there was no significant difference in expression of *HMGA2* between the partial and the complete form of the hydatidiform moles (PHM-CHM: $p = 0.552$, Welch t-test), nor between these and the hydropic abortions (PHM-HA: $p = 0.881$; CHM-HA: $p = 0.791$). Due to the relatively small number of samples, the non-availability of the gestational age in some cases and the small range of the ages, where these were available, gestational age was not considered in this comparison.

*HMGA2* expression in hydatidiform moles was not significantly different from the

expression in normal placentas (see chapter 3.4.) from the same age window (five to ten weeks, CGA), neither for all placentas ($p$ = 0.0556, Welch t test) nor for the spontaneous abortions subgroup ($p$ = 0.0941) or joint subgroups of specimens gathered after induced abortion and after delivery ($p$ = 0.0607). Only expression data from three moles could be used for these tests. Also, when comparing the *HMGA2* expression in hydatidiform mole subgroups and the hydropic abortions with the expression in normal placentas (all cases), no significant differences could be found (PHM: $p$ = 0.665; CHM: $p$ = 0.759; HA: $p$ = 0.819, Welch t test).

### 3.5.3. Immunohistological analysis

With the exception of cases 5 and 6, all samples were tested for the localization of HMGA2 protein. Level and pattern of distribution follows closely the one from the non-hydropic samples (see chapter 3.4.). There was no striking difference between hydropic abortions, PHMs and CHMs, only slightly less intense signals in comparison to the non-hydropic samples were noticeable, which is consistent with the results from the qRT-PCR (figure 3).

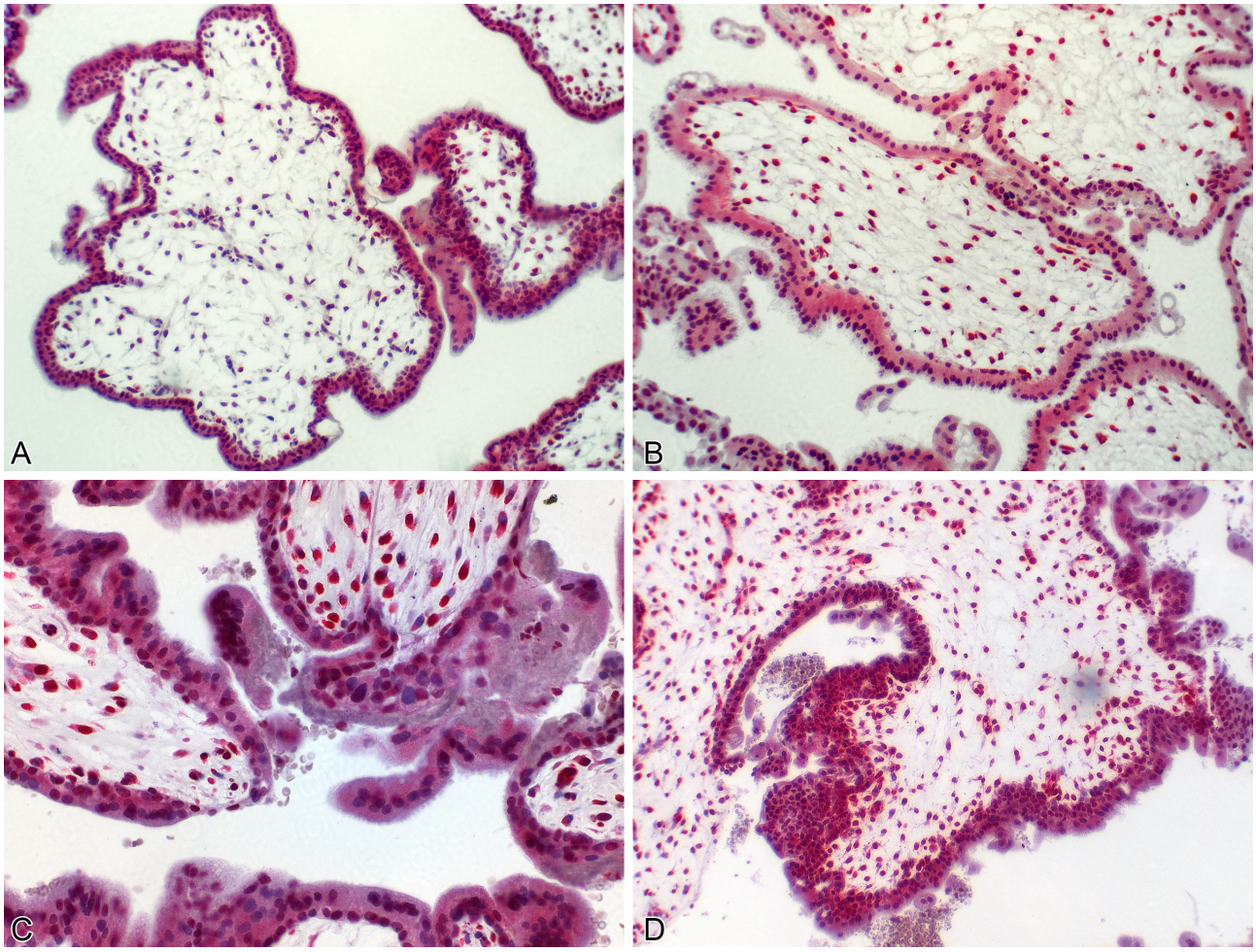



**Figure 3**: Immunoreactivity for HMGA2 in HA, PHM, and CHM samples.

(A): triploid PHM, gestational age not available; (B): tetraploid PHM, nine weeks calendar gestational age; (C): HA, eight weeks calendar gestational age; (D): CHM, six weeks calendar gestational age. Except for a seemingly less intense signal the pattern of the staining is very similar to those of the non-hydropic placentas of comparable age. Pictures (A) to (D) correspond to sample numbers 1, 2, 3, and 8, respectively, in the investigation (see also table 1C).

## 3.6. Quantitative analysis of *HMGA2* in testicular cancer

**Publication IV: *HMGA2* expression distinguishes between different types of post-pubertal testicular germ cell tumours cancer (Kloth, Gottlieb *et al*., The Journal of Pathology: Clinical Research, 2015, accepted)**

Human germ cell tumors can be classified into five main groups, distinguished by several criteria such as histology and developmental origin (Oosterhuis, Looijenga, 2005). Hydatidiform moles are of type V, type II consists of testicular seminomatous and non-seminomatous GCTs. As mentioned before, hydatidiform moles of the uterus can lead to the very aggressive choriocarcinoma. This type of tumor can also develop in testicular tissue of the male. After elucidating the expression of *HMGA2* in context of the developmental stage in the placenta, this study dealt with the presumed differences in expression of the gene in the subtypes of post-pubertal germ cell tumors of the testis.

qRT-PCR data from 59 samples and immunohistochemical analysis of 23 of these specimens revealed significant differences in expression pattern. Seminomas and embryonal carcinomas showed expression levels very close to normal tissue, yolk sac tumors a distinct upregulation. In teratomas, expression varied considerably between samples and could also differ within one specimen. Due to the small amount of choriocarcinomas, no final conclusion can be drawn, but results indicate a moderate expression. Statistical analysis revealed significant differences between the subgroups in a linear regression analysis of real-time PCR values. Additionally, applying the Wilcoxon two-sample rank sum test, significant differences were found between yolk sac tumors and all other samples using data from the immunohistochemical analysis. In all cases qRT-PCR results were in concordance with those from the immunohistochemical analysis. It could also be shown, that the antibody specific for HMGA2 might serve as a tool in pathological assessment of samples in clinical application.

## IV

## *HMGA2* expression distinguishes between different types of post-pubertal testicular germ cell tumours

Lars Kloth, Andrea Gottlieb, Burkhard M. Helmke, Werner Wosniok, Thomas Löning, Gazanfer Belge, Kathrin Günther, and Jörn Bullerdiek

The Journal of Pathology: Clinical Research 2015, accepted

Own contribution:

Study design with Jörn Bullerdiek and Andrea Gottlieb

Execution and analysis of the study except for immunohistochemistry

Statistics with Werner Wosniok and Andrea Gottlieb

Writing of the manuscript with Andrea Gottlieb

Lars Kloth and Andrea Gottlieb contributed equally to this paper.

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**Original Article**

# *HMGA2* expression distinguishes between different types of postpubertal testicular germ cell tumour

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## Abstract

**The group of postpubertal testicular germ cell tumours encompasses lesions with highly diverse differentiation – seminomas, embryonal carcinomas, yolk sac tumours, teratomas and choriocarcinomas. Heterogeneous differentiation is often present within individual tumours and the correct identification of the components is of clinical relevance. *HMGA2* re-expression has been reported in many tumours, including testicular germ cell tumours. This is the first study investigating *HMGA2* expression in a representative group of testicular germ cell tumours with the highly sensitive method of quantitative real-time PCR as well as with immunohistochemistry. The expression of *HMGA2* and *HPRT* was measured using quantitative real-time PCR in 59 postpubertal testicular germ cell tumours. Thirty specimens contained only one type of tumour and 29 were mixed neoplasms. With the exception of choriocarcinomas, at least two pure specimens from each subgroup of testicular germ cell tumour were included. In order to validate the quantitative real-time PCR data and gather information about the localisation of the protein, additional immunohistochemical analysis with an antibody specific for HMGA2 was performed in 23 cases. Expression of *HMGA2* in testicular germ cell tumours depended on the histological differentiation. Seminomas and embryonal carcinomas showed no or very little expression, whereas yolk sac tumours strongly expressed HMGA2 at the transcriptome as well as the protein level. In teratomas, the expression varied and in choriocarcinomas the expression was moderate. In part, these results contradict data from previous studies but HMGA2 seems to represent a novel marker to assist pathological subtyping of testicular germ cell tumours. The results indicate a critical role in yolk sac tumours and some forms of teratoma.**

**Keywords:** testicular germ cell tumour; HMGA2; seminoma; embryonal carcinoma; yolk sac tumour; teratoma; choriocarcinoma; qRT-PCR; immunohistochemistry; biomarker

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[†]These authors contributed equally to this paper.

[‡]Conflict of interest: The authors declare that they have no competing interest.

## Introduction

Testicular germ cell tumours (TGCTs) are relatively rare, but in many countries they represent the most prevalent cancer in men between 15 and 40 years of age [1]. For unknown reasons its incidence has increased significantly over recent decades in many populations globally [1,2]. Nevertheless, the worldwide frequency varies considerably between different races and countries, with several European countries showing the highest incidences [1,3,4]. In these regions, up to one in 200 men is affected [3]. The

 

assumed originating cells are primordial germ cells, which undergo malignant transformation thus becoming an intratubular germ cell neoplasia undifferentiated (ITGCNU), formerly referred to as testicular intraepithelial neoplasia or carcinoma *in situ* [reviewed in [5,6]]. In almost all cases of ITGCNU, a TGCT with invasive growth eventually develops [reviewed in [7,8]]. TGCTs are divided into pure seminoma (∼50–54%) and nonseminoma (ca. 46–50%) subgroups. The latter also contains mixed tumours [9,10]. This subtyping is of clinical and prognostic relevance [10–12]. Nonseminomas display different degrees of differentiation from embryonal carcinoma (EC) to mature teratoma and display embryonic and extraembryonic differentiation [8,13]. Mixed forms of two or more nonseminomas, or seminoma and nonseminoma, are common: between 13 and 54% of tumours have been reported to contain mixed histology [14,15]. Although a panel of immunohistochemical biomarkers helps to differentiate tumour subgroups, diagnosis can still be a challenge [16].

High-mobility group AT-hook (HMGA) proteins are small and highly charged, consisting of three DNA-binding domains and an acidic carboxy-terminal tail. As architectural transcription factors they lack intrinsic transcription factor capacity but interact with nuclear proteins and enhance or silence transcription through changes in chromatin structure [reviewed in [17,18]]. There are four known HMGA proteins in humans (HMGA1a, HMGA1b, HMGA1c and HMGA2), encoded by two genes [reviewed in [19,20]]. High *HMGA* expression has been detected at embryonic and foetal stages in mammalian tissues. Conversely, no or only very low *HMGA2* expression has been identified in adult tissue; slightly higher levels have been reported for *HMGA1* in some tissues [21–25]. The reactivation of *HMGA* expression has been reported for a multitude of tumours [reviewed in [19,26,27]. Chieffi et al. [28] found *HMGA2* to be critically involved in spermatogenesis in mice. Furthermore, Di Agostino *et al*. [29] found that *HMGA2* interacts with Nek2 in a MAPK-dependent manner in mouse spermatogenesis. In addition to the participation in normal testicular processes, *HMGA2* has also been suggested as a marker for testicular cancer [30 and reviewed in [5,31,32]]. Franco *et al*. [30] showed moderate to high expression of *HMGA2* in ECs and yolk sac tumours (YSTs).

The aim of this study was to investigate the role of *HMGA2* in postpubertal germ cell tumours of the testis. For the first time, highly sensitive quantitative real-time PCR (qRT-PCR) has been applied in combination with immunodetection, to allow more distinctive differentiation of expression levels of *HMGA2* in the subgroups. Another key aspect was determining whether protein level could serve as a diagnostic marker for clinical application.

## Methods

### Tissue samples

Formalin-fixed paraffin-embedded (FFPE) tumour tissue and snap-frozen samples of normal testis were collected at the Department of Pathology, Albertinen Hospital, Hamburg, Germany, the Department of Pathology, Clinical Centre Bremen-Mitte, Bremen, Germany, and the Institute of Pathology, Elbe Clinic Stade-Buxtehude, Germany. Additional FFPE samples were collected under the supervision of the Leibniz Institute for Prevention Research and Epidemiology, Bremen, Germany. Pathological examinations were performed after haematoxylin and eosin staining of the samples for diagnostic purposes. In complex cases, additional immunostaining with antibodies specific for PLAP, OCT4, CD30, CD117, glypican 3, AFP and $\beta$-HCG was conducted according to the relevant pathology department's protocol. FFPE tissue samples from 59 postpubertal patients with TGCT and three snap-frozen normal testis tissues were examined in the study. Histology was re-evaluated by three of the authors (B.H., T.L. and K.B.) according to the WHO classification. Histological subgroups were: 12 pure seminomas, three mixed tumours with a predominant component of seminoma, 10 pure ECs, 13 mixed tumours with a predominant component of EC, three mixed tumours with two equally predominant components of EC/teratoma or EC/YST, two YSTs, three mixed tumours with a predominant component of YST, six pure teratomas, seven mixed tumours with a predominant component of teratoma (see also Table 1). All samples investigated were initially taken for diagnostic purposes and secondarily used for the present study. Samples were deidentified before their use in this study, in line with the rules of the Helsinki declaration. The study was approved by the local ethics committee (Ärztekammer Bremen, reference number 371).

### RNA isolation

Depending on the size of the embedded tissue, FFPE blocks were cut into six to eight sections of 5 $\mu$m for each sample using a microtome. Total RNA isolations were performed using the innuPREP Micro RNA Kit (Analytik Jena AG, Jena, Germany) for

 

Table 1. Overview of all testicular cancer samples

| Case | RQ | Sample composition (values relative to cancerous content) | | | | | | Normal tissue, absolute value (%) | Immunohistochemistry | Patient's age | Tumour size (cm) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Seminoma (%) | EC (%) | YST (%) | Teratoma (%) | CC (%) | Undetermined (%) | | | | |
| HT01 | 7,998 | 0 | 4 | 4 | 92 | 0 | 0 | 75 | − | 32 | 2,8 |
| HT02 | 3,617 | 100 | 0 | 0 | 0 | 0 | 0 | 11 | − | 46 | 6,5 |
| HT03 | 9,182 | 0 | 50 | 10 | 40 | 0 | 0 | 70 | − | 18 | 2,7 |
| HT04 | 0.763 | 100 | 0 | 0 | 0 | 0 | 0 | 70 | − | 60 | 1,6 |
| HT05 | 0.507 | 100 | 0 | 0 | 0 | 0 | 0 | 90 | − | 35 | 1,3 |
| HT06 | 0.901 | 0 | 100 | 0 | 0 | 0 | 0 | 80 | − | 21 | 2.6 |
| HT07 | 3.689 | 0 | 100 | 0 | 0 | 0 | 0 | 70 | + | 29 | 1.9 |
| HT08 | 0.312 | 100 | 0 | 0 | 0 | 0 | 0 | 15 | − | 31 | 4.5 |
| HT09 | 130.584 | 0 | 40 | 40 | 0 | 0 | 20 | 25 | + | 39 | 3.5 |
| HT10 | 46.510 | 0 | 0 | 0 | 70 | 30 | 0 | 50 | + | 23 | 6 |
| HT11 | 0.865 | 100 | 0 | 0 | 0 | 0 | 0 | 30 | − | 55 | 1.5 |
| HT12V | 1.808 | 0 | 0 | 0 | 100 | 0 | 0 | 20 | − | 23 | 1.8 |
| HT13 | 0.234 | 100 | 0 | 0 | 0 | 0 | 0 | 80 | − | 39 | 1.5 |
| HT14 | 0.438 | 60 | 40 | 0 | 0 | 0 | 0 | 30 | − | 18 | 2 |
| HT15 | 1.624 | 0 | 100 | 0 | 0 | 0 | 0 | 85 | − | 27 | 2 |
| HT16 | 0.733 | 100 | 0 | 0 | 0 | 0 | 0 | 20 | − | 41 | 5.5 |
| HT17 | 111.724 | 0 | 40 | 40 | 0 | 20 | 0 | 17 | + | 28 | 2.5 |
| HT19 | 0.1* | 100 | 0 | 0 | 0 | 0 | 0 | 70 | + | 38 | 2.8 |
| HT20 | 1.765 | 100 | 0 | 0 | 0 | 0 | 0 | 90 | + | 50 | 1.5 |
| HT22 | 6.922 | 0 | 100 | 0 | 0 | 0 | 0 | 64 | + | 53 | 1.5 |
| HT23 | 1.880 | 0 | 100 | 0 | 0 | 0 | 0 | 90 | − | 35 | 3.5 |
| HT24 | 10.833 | 0 | 4 | 0 | 96 | 0 | 0 | 75 | − | 28 | 0.9 |
| HT25 | 1.041 | 75 | 20 | 5 | 0 | 0 | 0 | 56 | + | 20 | 2.8 |
| HT26 | 0.681 | 100 | 0 | 0 | 0 | 0 | 0 | 10 | − | 26 | 3.3 |
| HT27 | 0.143 | 100 | 0 | 0 | 0 | 0 | 0 | 30 | + | 39 | 6.5 |
| HT28 | 0.322 | 100 | 0 | 0 | 0 | 0 | 0 | 85 | − | 46 | 2.4 |
| HT29 | 0.873 | 0 | 0 | 0 | 100 | 0 | 0 | 15 | + | 21 | 2 |
| HT30 | 74.481 | 0 | 0 | 20 | 80 | 0 | 0 | 38 | + | 66 | 4 |
| HT31 | 17.595 | 0 | 90 | 5 | 0 | 5 | 0 | 43 | − | 37 | 6.5 |
| HT32 | 31.621 | 0 | 60 | 30 | 0 | 5 | 5 | 40 | − | 24 | 8 |
| HT33 | 32.310 | 0 | 80 | 10 | 0 | 10 | 0 | 25 | − | 23 | 4 |
| HT34 | 6.474 | 0 | 96 | 4 | 0 | 0 | 0 | 33 | − | 33 | 1.8 |
| HT35 | 0.379 | 0 | 0 | 75 | 25 | 0 | 0 | 38 | + | 35 | 4 |
| HT36 | 50.418 | 0 | 0 | 100 | 0 | 0 | 0 | 26 | − | 36 | 1.5 |
| HT37 | 61.414 | 0 | 80 | 20 | 0 | 0 | 0 | 47 | − | 22 | 3.4 |
| HT38 | 1.594 | 0 | 0 | 5 | 95 | 0 | 0 | 20 | − | 35 | 2.6 |
| HT39 | 7.565 | 0 | 90 | 10 | 0 | 0 | 0 | 50 | − | 47 | 3 |
| HT40 | 1.403 | 0 | 100 | 0 | 0 | 0 | 0 | 11 | + | 30 | n.a. |
| HT41 | 14.691 | 0 | 59 | 5 | 35 | 1 | 0 | 11 | − | 40 | 1.2 |
| HT42 | 109.424 | 0 | 0 | 100 | 0 | 0 | 0 | 25 | + | 38 | 6 |
| HT43 | 99.796 | 0 | 5 | 10 | 85 | 0 | 0 | 15 | + | 31 | 3.5 |
| HT44 | 147.842 | 0 | 40 | 18 | 40 | 2 | 0 | 30 | + | 24 | 3.2 |
| HT45 | 6.866 | 0 | 0 | 0 | 100 | 0 | 0 | 70 | + | 43 | 5.5 |
| HT46 | 18.707 | 20 | 60 | 20 | 0 | 0 | 0 | 40 | − | 19 | 2.7 |
| HT47 | 15.294 | 0 | 95 | 5 | 0 | 0 | 0 | 50 | − | 48 | 2 |
| HT48 | 2.960 | 0 | 100 | 0 | 0 | 0 | 0 | 80 | − | 23 | 1 |
| HT49 | 0.897 | 0 | 0 | 0 | 100 | 0 | 0 | 50 | − | 24 | 1.8 |
| HT50 | 0.853 | 0 | 0 | 0 | 100 | 0 | 0 | 80 | − | 21 | 1.5 |
| HT51 | 626.427 | 0 | 10 | 20 | 70 | 0 | 0 | 20 | + | 40 | 4.5 |
| HT52 | 230.972 | 75 | 5 | 20 | 0 | 0 | 0 | 30 | + | 19 | 3 |
| HT53 | 28.455 | 0 | 90 | 10 | 0 | 0 | 0 | 30 | + | 18 | n.a. |
| HT54 | 130.314 | 0 | 0 | 95 | 5 | 0 | 0 | 41 | − | 43 | 3 |
| HT55 | 13.557 | 0 | 95 | 5 | 0 | 0 | 0 | 22 | + | 23 | 3 |
| HT56 | 6.136 | 0 | 100 | 0 | 0 | 0 | 0 | 29 | − | 29 | 2.1 |
| HT57 | 2.301 | 0 | 0 | 0 | 100 | 0 | 0 | 20 | − | n.a. | 3.5 |
| HT58 | 3.549 | 0 | 100 | 0 | 0 | 0 | 0 | 33 | − | 43 | 2 |
| HT59 | 6.824 | 0 | 100 | 0 | 0 | 0 | 0 | 38 | − | 30 | 4.5 |
| HT62 | 3.733 | 0 | 81 | 10 | 9 | 0 | 0 | 70 | + | 27 | n.a. |
| HT63 | 119.312 | 0 | 50 | 50 | 0 | 0 | 0 | 26 | + | 38 | 2.5 |

RQ, relative quantification; *HMGA2* expression, EC, embryonal carcinoma; YST, yolk sac tumour; CC, choriocarcinoma; Immunhistochemistry, sample was used in HMGA2-specific immunhistochemistry investigation; *: set value, expression below detection level (see text for further explanation), n.a., not available.

RNA isolation according to the manufacturer's instructions, with the following modifications: Lysis of the paraffin sections preceding RNA isolation was conducted using TLS-Lysis Solution and Proteinase K from the innuPREP DNA Micro Kit (Analytik Jena AG, Jena, Germany) without prior deparaffinisation. Sections were incubated for 1 h at 60°C and 15 min at 80°C.

### cDNA-synthesis and quantitative real-time RT-PCR

RNAs were reverse-transcribed into cDNA by M-MLV Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). Real-time PCR was performed using the Applied Biosystems 7300 sequence detection system, software 1.2.3, according to the Taq-Man Gene Expression Assay Protocol (Applied Biosystems, Darmstadt, Germany) in 96-well microtitre plates with a total volume of 20 $\mu$l. For the TaqMan gene expression assay for *HMGA2* (assay number Hs00171569, Applied Biosystems, Foster City, USA), each reaction consisted of 2 $\mu$l of cDNA reverse transcribed from 25 ng of total RNA, 10 $\mu$l of TaqMan Universal PCR Master Mix (Applied Biosystems), 1 $\mu$l of TaqMan assay and 7 $\mu$l of $ddH_2O$. For the *HPRT* assay, using HPRT FP and HPRT RP primers [33], each reaction consisted of 2 $\mu$l of cDNA reverse transcribed from 25 ng of total RNA, 10 $\mu$l of TaqMan Universal PCR Master Mix, 600 nM (1.2 $\mu$l) of forward and reverse primers, 200 nM (0.2 $\mu$l) of probe [33] and 5.4 $\mu$l of $ddH_2O$. Thermal cycling conditions were 2 min at 50°C followed by 10 min at 95°C, 50 cycles at 95°C for 15 s and 60°C for 1 min. In each run, a negative control of previous cDNA synthesis (missing reverse transcriptase) was included for each sample and a nontemplate control of amplification and a nontemplate control of previous cDNA synthesis were included for each plate.

All testing reactions were performed in triplicate. Considering the expression range of *HMGA2*, *HPRT* was chosen as the endogenous control as generally suggested by de Kok *et al*. [34], and as used for testicular samples by McIntyre *et al*. [35], Looijenga *et al*. [36] and Wermann *et al*. [37]. The $C_T$ values of both genes were in concordance (*HMGA2*: 21,914–36,006; *HPRT*: 23,421–37,459). As recommended for FFPE samples [38] the fragment sizes amplified by both assays were small, ranging between 65 and 80 bp; validation of these values was performed via gel electrophoresis of the PCR amplicons (data not shown). Relative quantity (RQ) was calculated using the ddCT method [39]. Snap frozen tissue of normal testis was tested against FFPE from the same sample giving highly comparable results. Because of disposability of snap frozen normal testis tissue, the average of three such tissues was used as calibrator.

### Immunohistochemical analysis

Slides utilized for the immunohistochemical analysis were produced using cuts directly adjacent to those used for the qRT-PCR investigation. Immunohistochemical staining for HMGA2 (rabbit polyclonal anti-HMGA2-P3, Biocheck, Inc., Forster City, USA) was performed using a detection kit (DAKO ChemMate; DAKO, Glostrup, Denmark) and a semiautomated stainer (DAKO; TechMate) according to the specifications of the manufacturer. For antigen retrieval, the slides were treated in a PT Link module (DAKO) using the EnVision$^{TM}$ FLEX Target Retrieval Solution, low pH (DAKO). The antibody dilution used was 1:1000. Term placenta was used as a positive control whereas negative control was performed by omission of the primary antibody.

Interpretation of HMGA2 staining was done using a Zeiss Axioplan (Carl Zeiss Microscopy GmbH, Göttingen, Germany) microscope. Immunoreactivity in the nucleus was considered positive (although perinuclear granulation in cytoplasm was observed occasionally). In each slide, three to five high-power fields were rated. Staining extent was scored by multiplying intensity of staining (0: no staining, 0.5: very weak, 1: weak, 1.5: weak-moderate, 2: moderate, 2.5: moderate-strong and 3: strong) by percentage of stained tumour cells. Lack of available tissue was one of the reasons why we did not perform IHC in all cases analysed by qRT-PCR.

### Statistical analysis

RQ values and immunohistochemical scores were described by number of values, arithmetic means, standard deviations and minimum and maximum. Boxplots were used to summarize the distribution of data values. Averages of immunohistochemical scores were compared by the Wilcoxon two-sample rank sum test. The ability of the RQ value to discriminate between tumour subgroups was quantified for all pairs of tumour subgroups by sensitivity and specificity, obtained from Receiver-Operator-Characteristics (ROC) analysis, thereby using a normal approximation of the empirical data. This analysis was performed for all samples containing only a single type of tumour. The relationship between lg(RQ) and the proportion of tumour components – these expressed as proportion of the total section area – was analysed by linear regression. An intercept was omitted from the

 

Table 2. *HMGA2* expression in pure tumours

| Case | RQ | Type of tumour |
|---|---|---|
| HT02 | 3.617 | seminoma |
| HT04 | 0.763 | seminoma |
| HT05 | 0.507 | seminoma |
| HT08 | 0.312 | seminoma |
| HT11 | 0.865 | seminoma |
| HT13 | 0.234 | seminoma |
| HT16 | 0.733 | seminoma |
| HT19 | 0.1* | seminoma |
| HT20 | 1.765 | seminoma |
| HT26 | 0.681 | seminoma |
| HT27 | 0.143 | seminoma |
| HT28 | 0.322 | seminoma |
| HT06 | 0.901 | EC |
| HT07 | 3.689 | EC |
| HT15 | 1.624 | EC |
| HT22 | 6.922 | EC |
| HT23 | 1.880 | EC |
| HT40 | 1.403 | EC |
| HT48 | 2.960 | EC |
| HT56 | 6.136 | EC |
| HT58 | 3.549 | EC |
| HT59 | 6.824 | EC |
| HT36 | 50.418 | YST |
| HT42 | 109.424 | YST |
| HT12V | 1.808 | teratoma |
| HT29 | 0.873 | teratoma |
| HT45 | 6.866 | teratoma |
| HT49 | 0.897 | teratoma |
| HT50 | 0.853 | teratoma |
| HT57 | 2.301 | teratoma |

RQ, relative quantification; EC, embryonal carcinoma; YST, yolk sac tumour; *, set value; expression below detection level (see text for further explanation).

regression equation, because a tumour proportion of zero is by definition associated with $\lg(RQ) = 0$. To allow for the logarithmic transformation of all values, the RQ of zero observed in one case was replaced by $RQ = 0.1$. This value still lies below the smallest observed RQ value. All cases were included in this analysis. A $p$ value of less than 0.05 was considered significant, a $p$ value of less than 0.001 highly significant. Statistical analyses were undertaken using the SAS/STAT and SAS/GRAPH software (version 9.2 for Windows, copyright 2002–2008 SAS Institute Inc.), and the R software [40].

## Results

### qRT-PCR analysis

Fifty-nine FFPE samples of human TGCTs were tested for the expression of *HMGA2* (Table 1). Of these tumours, 30 were pure tumours (12 seminomas, 10 ECs, two YSTs and six teratomas), 29 were mixed GCTs. These were accompanied by three snap-frozen normal testicular samples used for normalisation. Overall, the samples showed an *HMGA2* expression level between 0.143 and 626.427: this relates to a range of about 1–4381. For one sample, the expression was 0, ie the expression was below the detection limit.

Table 3. *HMGA2* expression in pure tumours by group

| Type of tumour | *n* | Average | St dev |
|---|---|---|---|
| Seminoma | 12 | 0.904 | 1.004 |
| EC | 10 | 3.589 | 2.292 |
| YST | 2 | 79.921 | 41.724 |
| Teratoma | 6 | 2.266 | 2.332 |

*n*, number of cases; St dev, standard deviation; EC, embryonal carcinoma; YST, yolk sac tumour.

Focussing on the samples with only one tumour subgroup, there was a clear classification between the groups (Tables 2 and 3, Figure 1). Seminomas showed the lowest values; with two exceptions, all measured data were below the expression in normal tissue. ECs and teratomas showed slightly elevated levels, while the levels expressed by YSTs were by far the highest. This clustering could be visualized by aligning the samples by level of expression (Figure 2, including the mixed tumours).

To statistically validate the visual impression on discriminatory ability, ROC analyses were performed (Table 4). Comparisons involving YST showed a sensitivity of at least 0.988 and a specificity of 0.997, indicating a clear distinction from the other tumour subgroups. These numbers, however, must be treated with caution, since the YST group consisted of only two samples. In addition to the comparison of individual groups, seminomas were tested against all other subgroups. This analysis indicated that seminomas and nonseminomas were separated moderately well by real-time *HMGA2* expression data alone with a sensitivity of 0.912 and a specificity of 0.680.

To analyse qRT-PCR data from samples with two or more tumour components and to accommodate for varying percentages of normal tissue content, a linear regression was calculated between the logarithmic RQ and the tumour components (Table 5, Figure 3). Comparison of observed and predicted RQ values showed good agreement; in particular, no indication of systematic deviation was identified. The model achieved an adjusted coefficient of determination of 0.6625 with a $p$ value of $1.112*10^{-12}$, indicating *HMGA2* expression is dependent on the tumour subgroup. With the exception of seminomas, each subgroup's contribution turned out to be significant or highly significant (see also Table 5). There are four values with large differences between observed and

 

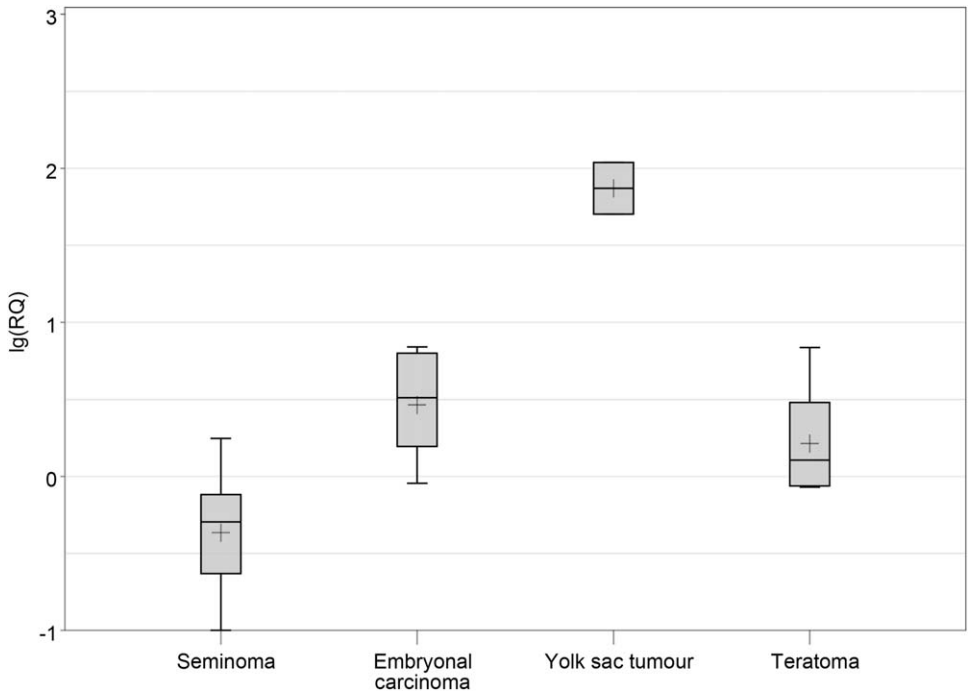


**Figure 1.** *HMGA2* expression in pure tumours. Boxplots for the relative quantification of *HMGA2* expression in TGCTs. Tumour type at *x*-axis, logarithmic RQ at *y*-axis. Boxes contain the central 50% of all values and a bar at the position of the median, whiskers extend to the extreme values or to 1.5 * box height, whichever is smaller. The plus sign shows the arithmetic mean.

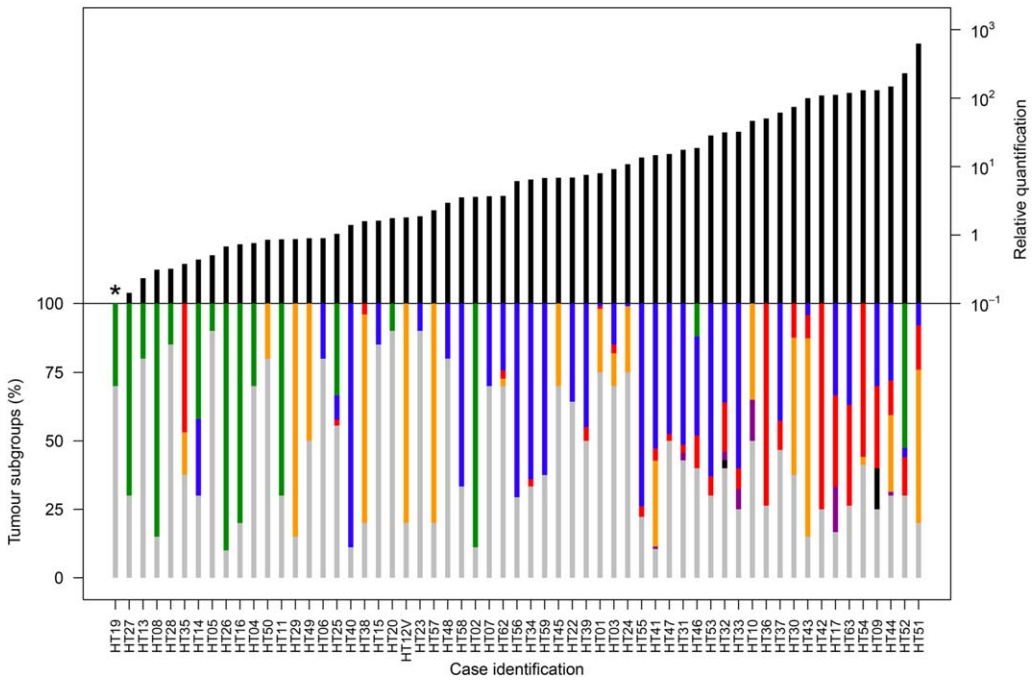


**Figure 2.** Overview of all testicular cancer samples. Bar plot with RQ in the upper part, tumour composition in the lower part. Samples are arranged by level of *HMGA2* expression. *: set value, expression below detection level (see text for further explanation). Colour code for the tumour composition: green: seminoma, blue: embryonal carcinoma, red: yolk sac tumour, orange: teratoma, purple: choriocarcinoma, black: undetermined, grey: normal tissue.

 

Table 4. ROC analysis of pure tumours

| Type of tumour | *n* | | Seminoma | EC | YST | Teratoma |
|---|---|---|---|---|---|---|
| Seminoma | 12 | sens. | | 0.868 | 0.998 | 0.581 |
| | | spec. | | 0.815 | 0.999 | 0.828 |
| EC | 10 | sens. | | | 0.988 | 0.883 |
| | | spec. | | | 0.997 | 0.371 |
| YST | 2 | sens. | | | | 0.996 |
| | | spec. | | | | 0.998 |
| Teratoma | 6 | sens. | | | | |
| | | spec. | | | | |

*n*, number of cases; sens., sensitivity; spec., specificity; EC, embryonal carcinoma; YST, yolk sac tumour.

predicted expression. HT35 is a clear outlier, with measured expression far below the expected value. As the sample was composed of YST and teratoma, taking the overall results into consideration, much higher expression of *HMGA2* had been expected. HT51 and HT52 showed values higher than expected considering their composition. HT19 is the sample with the set value. In all cases, qRT-PCR data were confirmed by immunohistochemical analysis (see below).

### Immunhistological analysis

In 23 cases, the section of the FFPE block following those used for qRT-PCR analysis was investigated for HMGA2 protein expression patterns. Since 14 samples contained two or more histologically different areas, 45 immunohistochemical scores were gathered (Figure 4). Concordant with results from qRT-PCR, the HMGA2 scores in seminomas were nearly zero (mean: 0.0375). One seminoma (HT20) showed focally strong immunoreactivity; all the others were negative. No or very weak staining was observed in EC components, whereas a wide range was observed in teratoma components. There was a tendency for immature structures to be positive, whereas mature teratomas were negative. Exceptions were observed, eg mature glandular structures as goblet cells were often, but not always, strongly positive. On the other hand, primitive neuroepithelium showed weak staining; muscular structures were negative. YST components were strongly positive. Two exceptions were found: one YST was negative (HT25: 5% YST, 75% seminoma, 20% EC) and one showed weak to moderate staining (HT35: 75% YST, 25% teratoma). Both also showed unexpectedly low HMGA2 expression by qRT-PCR. HT51 and HT52, both displaying very high qRT-PCR values, showed equally strong immunostaining. In choriocarcinomas (CCs) syncytiotrophoblasts as well as cytotrophoblasts showed weak to moderate staining (Figure 5, Supplemental Figure 1). HMGA2 expression was

Table 5. Linear regression analysis of the relationship between lg(RQ) and the proportion of tumour components

| Tumour component | Estimate | Standard error | *t* value | *p* value |
|---|---|---|---|---|
| Seminoma | −0.001496 | 0.003134 | −0.477 | 0.63502 |
| EC | 0.014816 | 0.002875 | 5.153 | $3.72*10^{-6}$ |
| YST | 0.030189 | 0.004875 | 6.192 | $8.38*10^{-8}$ |
| Teratoma | 0.01078 | 0.00327 | 3.297 | 0.00173 |
| CC | 0.06092 | 0.029689 | 2.052 | 0.04504 |

Estimate, estimate of the regression coefficient; positive values indicate an RQ value increasing with tumour proportion, *t* value, test statistic for the hypothesis 'Coefficient is zero'; *p* value, level of significance; EC, embryonal carcinoma; YST, yolk sac tumour; CC, choriocarcinoma. The model contains no intercept, as a proportion of zero is by definition associated with lg(RQ) = 0; data: all samples.

seen in 80–100% of syncytiotrophoblastic cells and in 60% of cytotrophoblast components. As we did not find syncytiotrophoblastic cells in our seminoma cases we can neither confirm nor exclude HMGA2 expression in this situation. In normal tissue, HMGA2 was detected in the cytoplasm of the spermatogonial cells. Nuclear expression was weak in spermatocytes and strong in spermatids. Spermatozoa were negative for the protein (Supplemental Figure 1).

A Wilcoxon two-sample rank sum test (Table 6) was conducted to evaluate the separation of tumour entities. Due to multiple testing, a corrected $\alpha = 0.005$ was used. Despite this restriction, significant differences were detected when comparing scores from YSTs with those from ECs and teratomas. Testing seminoma scores against nonseminoma scores resulted in a significant difference in protein level ($p = 0.0154$). Performing the same test with YST values against all other scores revealed a highly significant difference ($p = 3.821*10^{-6}$). Table 7 gives the results from both real-time PCR and immunostaining analysis.

## Discussion

An investigation using real-time PCR and immunohistology was performed to study the expression of *HMGA2* in all subgroups of TGCT. Overall, in comparison to normal tissue, seminomas showed a marginal decrease and ECs a slight upregulation. In teratomas, the expression level was variable and appeared to depend on cellular differentiation. CCs (syncytiotrophblastic giant cells and to a lesser extent cytotrophoblasts) and especially YSTs showed considerably increased expression. In normal testicular tissue, low *HMGA2* expression was detected by real-time PCR. This is most likely caused by temporarily high expression in cells involved in spermatogenesis.

 

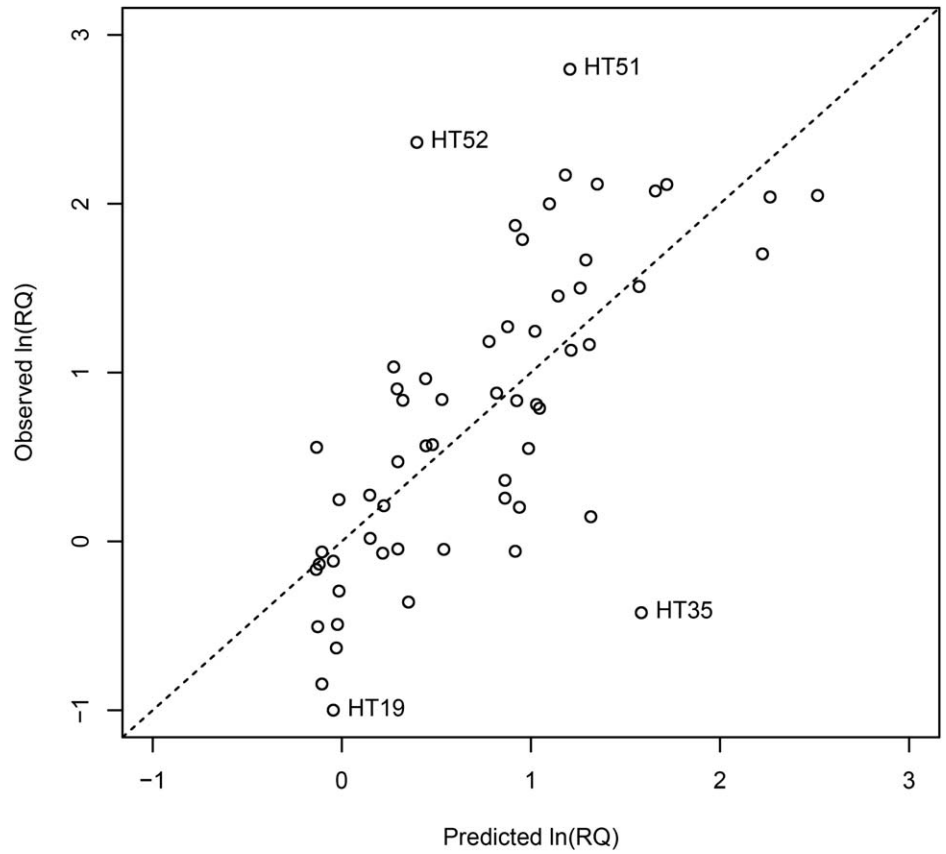


**Figure 3.** Linear regression analysis of the relationship between lg(RQ) and the proportion of tumour components. Predicted RQ at *x*-axis, observed RQ at *y*-axis, logarithmic scale. Each circle represents one sample. Outliers are marked by case identification (see text for details).

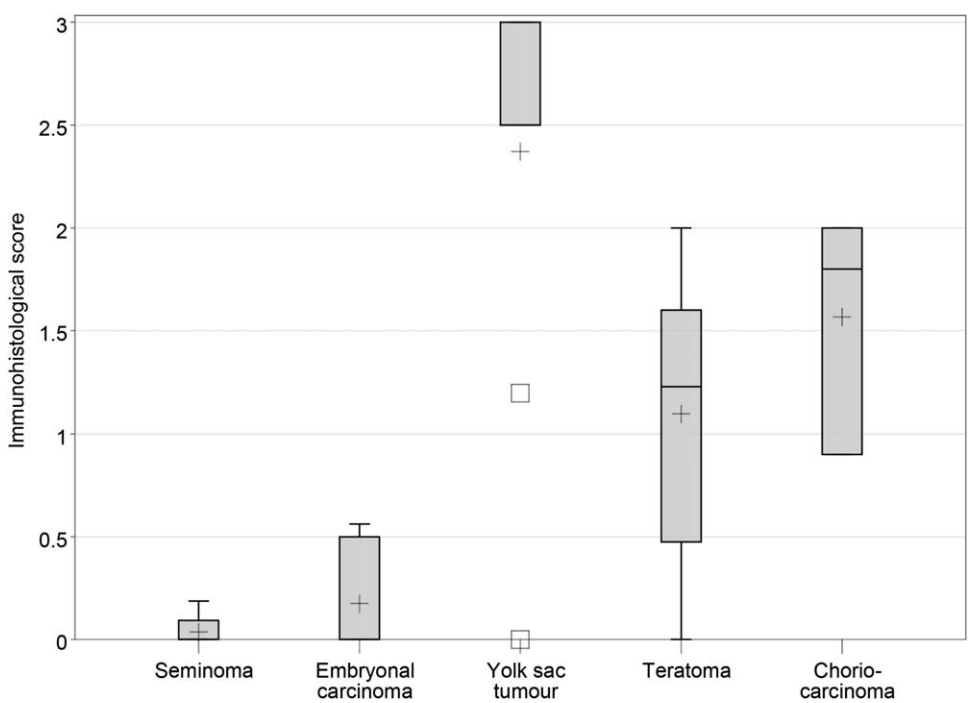


**Figure 4.** Immunostaining score by type of tumour. Boxes contain the central 50% of all values and a bar at the position of the median, whiskers extend to the extreme values or to 1.5* box height, whichever is smaller. The plus sign shows the arithmetic mean, the rectangles denote outliers.

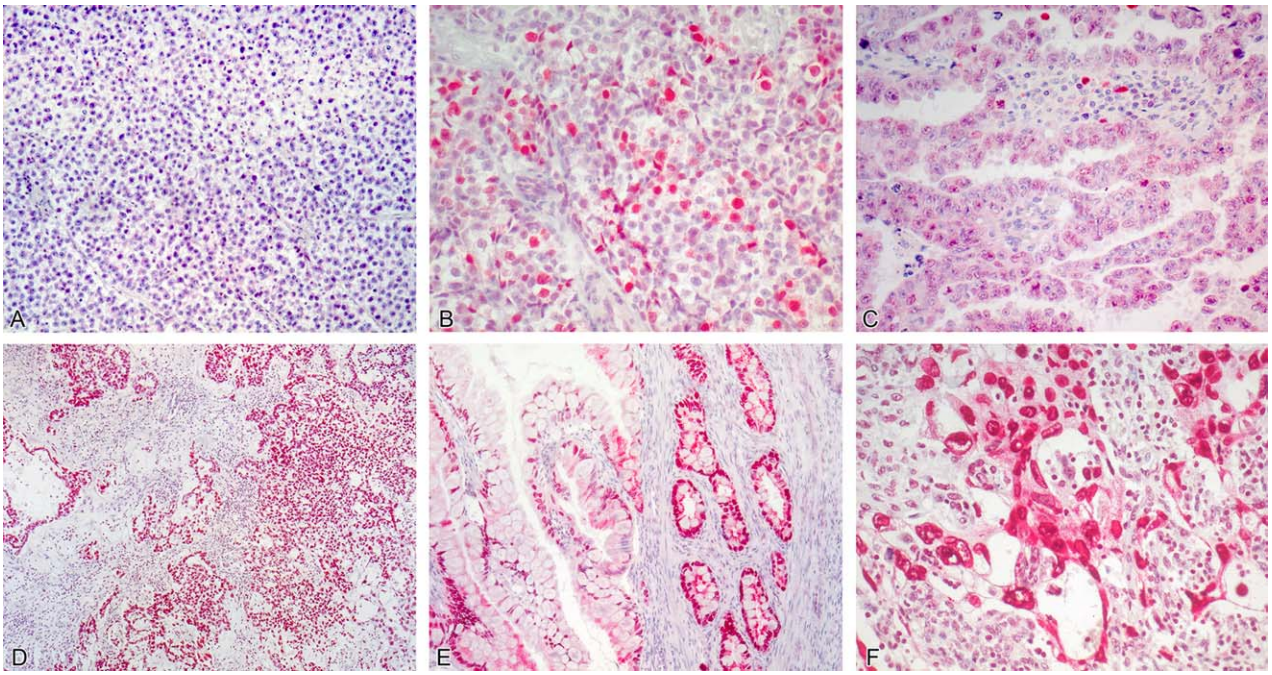


Figure 5. Immunoreactivity for HMGA2 in different tumour subgroups. A: seminoma; B: seminoma with focal HMGA2 reactivity; C: EC with weak granulation; D: YST, microcystic pattern; E: mature teratoma, heterogeneous staining intensity; F: CC intense staining in syncytiotrophoblasts. Original magnifications are given.

Contrary to normal tissue, no such pattern could be detected in most seminomas and immunostaining also showed no HMGA2 expression. Since all pure seminomas contained a percentage of normal tissue, it is plausible that the presence of *HMGA2* mRNA results from that portion. It is also possible that the very low expression detected by real-time PCR is below the threshold of immunohistochemical analysis. One seminoma with relatively high expression (>1) was also investigated using immunohistochemistry. Signals were restricted to one area where single HMGA2 positive cells were scattered in between negative seminoma cells (see Figure 5). One might hypothesize that further transformation of seminoma cells has taken place here. It is known that seminomas can progress into nonseminomas via EC [reviewed in [6]]. As HMGA2 staining in EC was in the range between not detectable and very weak, another type of transformation seems more likely. Nettersheim *et al*. [41] found that the seminoma cell line TCam-2 differentiates into mixed nonseminoma-like cell types without an intermediate step of EC after stimulation with growth-factors TGF-$\beta$1, EGF and FGF4. Gopalan *et al*. [42] also suggested a model in which teratoma, YST and CC develop directly from seminoma. Honecker *et al*. [43] found formations of germ cells developing in nonseminomas, but explicitly not in seminomas. This interesting finding remains to be investigated further. At this stage it can just be stated that we detected cells with enhanced self-renewing capacity within one seminoma.

Table 6. Wilcoxon two-sample test comparing the immunohistochemical score by group

| Type of tumour | *n* | Seminoma | EC | YST | Teratoma | CC |
|---|---|---|---|---|---|---|
| Seminoma | 5 | | 0.3219 | 0.0065 | 0.0223 | 0.0616 |
| EC | 14 | | | 0.0003 | 0.0054 | 0.0147 |
| YST | 14 | | | | 0.0045 | 0.0544 |
| Teratoma | 9 | | | | | 0.3294 |
| CC | 3 | | | | | |

*n*, number of values, EC, embryonal carcinoma; YST, yolk sac tumour; CC, choriocarcinoma. In each cell, the *p* value is stated (2-sided *t* approximation with continuity correction); $\alpha$ with Bonferroni correction: 0.005.

Table 7. Summary of qRT-PCR and immunohistochemical results

| Tumour component | *n* (qRT – PCR) | *n* (immunohisto) | *HMGA2* expression |
|---|---|---|---|
| Seminoma | 12 | 5 | 0 |
| EC | 10 | 14 | 0–+ |
| YST | 2 | 14 | +++ |
| Teratoma | 6 | 9 | 0–++ |
| CC | – | 3 | ++* |

*n* (qRT – PCR), number of values from real-time PCR; *n* (immunohisto), number of immunohistochemical scores; 0, no expression; (+), very weak expression; ++, moderate expression; +++, strong expression; *, preliminary deduction. Results obtained by linear regression analysis (see Table 5) were used in addition to qRT-PCR data and immunohistochemical scores for this summary.

 

ECs showed some variation, but always at a low to very low level. Tumours with YST components had a strong tendency towards high *HMGA2* expression, which was clearly confirmed by the immunohistochemical analysis. Teratomas showed a heterogeneous pattern of expression. It seemed that positivity depended on the type of teratoma structure. CC is the most uncommon type of TGCT [10]. Due to the limited availability of CCs, no qRT-PCR-data from homogeneously differentiated tumours could be gathered, and results from the immunohistochemical analysis were limited. Even though the samples present in this study showed a clear tendency, no definitive statement can be made for this subgroup at this point. These data partly confirm the results presented in Franco *et al*. [30]. Besides immunohistology, the authors used western blot analysis and RT-PCR. They also detected high expression of *HMGA2* in YSTs. For seminomas, no expression was reported, which coincides with our results from the immunohistochemical analysis. The residual presence of *HMGA2* expression is likely due to either the high sensitivity of this method, or to the presence of normal cells. In three of six pure teratomas in our study, qRT-PCR values were below one, the other three were slightly elevated. Taking the immunohistochemical analysis into account, and including mixed tumours with a teratoma component, teratomas turned out to be mostly positive, with only one exception of a pure mature teratoma without any positive staining. This is in contrast to the findings presented by Franco *et al*. [30] who did not find expression of *HMGA2* in teratomas. As they did not discriminate between immature and mature teratoma components, direct comparison cannot be performed. Even though it seems unlikely, it is not impossible that all of the 15 samples investigated by Franco *et al*. [30] were mature forms. Results for EC could not be reproduced: Franco *et al*. [30] reported one EC with moderate expression while 14 others showed high expression. This is in clear contrast to our results, for which we can not offer a straightforward explanation. Murray *et al*. [44] also reported positive results for EC. The authors investigated the LIN28/let-7 pathway in malignant germ cell tumours, and found a strongly negative correlation between LIN28 and let-7. As a minor aspect, the study also encompassed the analysis of the expression of *HMGA2*, but, due to the limited sample size of ECs ($n = 3$: one postpubertal, two paediatric), the results might not be representative for this particular group of neoplasms.

As HMGA2 is a nuclear protein, expression analysis generally focuses on nuclear staining [45–49]. Nevertheless, in the present study cytoplasmic positivity was observed occasionally in teratomas and to a lesser extent also in EC (Supplemental Figure 1). Other researchers have made similar observations in different tissues [47,50,51]. Taking into account these data and using a highly specific antibody, it seems less plausible that the cytoplasmic signal was artefactual. The task of developing an approach to clarify these findings remains.

Bearing a high mortality until the mid-1970s, today patients with a TGCT have a 5-year survival rate of 90–95% [52–54]. The remaining deaths are mostly due to chemoresistance of certain subgroups of TGCT: teratomas are benign-appearing, but metastases can form in 29% [15]. Mature teratomas have lost their embryonic features and are therefore completely resistant to cisplatin-based chemotherapy and other clinical treatment strategies [55]. After initial chemotherapy in patients with mixed TGCT with a portion of teratoma, teratoma can be found in the residual mass in 82% of cases [56]. CC metastasises early, therefore a high percentage of mixed tumours show a poor prognosis [15,57]. For several years, different studies found an amount of >50% EC to confer a higher risk for relapses [58]. Recently a follow-up study showed that the any presence of EC, independently of the amount, increases the relapse risk [59]. This illustrates the importance of determining the composition of the particular tumour. A proven set of antibodies for determination of the subgroups exists. Nevertheless, identification can pose a challenge for the pathologist [16,30], and a false diagnosis rate of 4–32% has been reported [60–62].

HMGA2 expression in YST turned out to be different from other types of TGCTs. To a somewhat lesser degree, immunohistochemical staining was also positive for (immature) teratoma components and CC. YST has a wide variety of growth patterns; it can be difficult to differentiate from seminomas, which is of therapeutic relevance [63]. AFP, the only immunohistochemical marker of YST for a long time, often shows only focal staining [15,64]. In recent years glypican-3, SALL4 and LIN28 have been established as diagnostic markers [15,64–68]. Glypican-3 has a higher sensitivity than AFP, but also shows focal staining [64,69]. This was confirmed in the present study. In most glandular growth patterns glypican-3 and HMGA2 showed identical expression. Yet much more HMGA2 positivity was observed in primitive reticular components with non-cohesive cells (Supplemental Figure 1G, 1I). Therefore HMGA2 staining seems to be more sensitive than glypican-3. HMGA2 shows expression (to a somewhat lesser degree) also in (immature) teratomas and CCs. Ota *et al*. [64] also found glypican-3

 

positivity in teratoma and CC. The specificity of HMGA2 and glypican-3 is therefore comparable for germ cell tumours. SALL4 and LIN28 are both sensitive markers. SALL4 is positive in all germ cell tumour subgroups including ITGCNU [65]. Therefore, it cannot be used to distinguish between different subgroups. LIN28 is sensitive for ITGCNU, seminomas, ECs and YSTs [66]. $\beta$-HCG is an established marker for CC, but as Lempiäinen *et al*. [70] showed recently, it can also be positive in ECs. Furthermore the authors found no expression in one of three pure CCs and two mixed TGCTs containing a CC component.

Our data suggest that the use of a HMGA2-specific antibody could be a sensible addition to existing markers and potentially help to decrease the rate of false diagnoses. A study composed of a larger number of FFPE and fresh-frozen samples, including a representative number of CCs, could bring this method even closer to clinical application. In addition, investigation of the expression of *HMGA2* in ovarian and extragonadal germ cell tumours would be of particular interest.

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## Authors' contributions

L.K. and J.B. conceived study. L.K., A.G. and J.B. designed study. L.K. carried out molecular genetic studies. A.G., L.K., B.H., T.L. and K.B. carried out immunohistological studies. B.H., T.L. and K.B. performed pathological analysis. B.H., T.L., K.B., G.B. and K.G. provided study material. W.W., L.K. and A.G. carried out statistical analysis. L.K. and A.G. wrote the manuscript. All authors revised the manuscript and had final approval of the submitted and published versions.

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## SUPPLEMENTARY MATERIAL ON THE INTERNET

Additional Supporting Information may be found in the online version of this article.

Supplemental Figure 1. Examples of HMGA2 immunoreactivity in normal testis tissue and different tumour subgroups. A: normal seminiferous tubules showing nuclear staining in spermatocytes and spermatids and weak cytoplasmic staining in spermatogonial cells, B: EC surrounded by YST, C: mature teratoma, glandular structure, D: immature teratoma, mesenchymal appearance, E: same as D, negative glypican-3 staining shows that no YST components are present, F: mature teratoma, glandular structures positive, muscular structures negative, G: glypican-3 staining in YST components restricted to glandular growth patterns, H: same as G, HMGA2 staining in YST is also strongly positive in primitive reticular components with noncohesive cells. Original magnifications are given.

 

# 4. Discussion

The interaction between cells within a multi-cellular organism is controlled through the temporally and quantitatively accurate expression of genes in each cell. Mutations in the genome can lead to aberrant gene products and abnormal levels of proteins, which in turn can cause diseases and syndromes. One of these diseases are lesions – hyperplasias, and benign and malignant tumors. In 2008, in Germany alone, almost 470,000 incidences of cancer and more than 215,000 cancer-related deaths in the population were counted (Robert Koch-Institut, 2012). These numbers emphasize the need to understand the cellular processes aiming at the treatment or even prevention of diseases.

Research by Rippe *et al*. (2003) uncovered a novel gene, which in a truncated form is suspected to be a cause of the development of thyroid adenomas, and they accordingly referred to it as *thyroid adenoma associated* (*THADA*) gene. As part of this thesis, *THADA* was found to be a marker for the dedifferentiation of thyroid tissue (Kloth *et al*., 2011). In a cohort of twelve subgroups of thyroid lesions and normal tissue samples, the most dedifferentiated anaplastic carcinomas showed a significant lower expression than all other samples combined (Kloth *et al*., 2011). Furthermore, a correlation with *sodium-iodide symporter* (*NIS)* was detected. *NIS* is considered a marker of thyroid differentiation (Ward *et al*., 2003; Li, Ain, 2010). Therefore, it was concluded that *THADA* may take part in maintaining the differentiation of follicular epithelium (Kloth *et al*., 2011). For treatment of thyroid lesions the status of differentiation is of great importance, since radioiodine uptake in tumors is no longer given when thyrocytes are progressively dedifferentiated and do not present iodide transporters on the basolateral membrane of the cells. Congruously, anaplastic thyroid tumors, as well as dedifferentiated forms of papillary and follicular neoplasms do not respond well to conventional radio- or chemotherapy (Vivaldi *et al*., 2009).

Thyroid-stimulating hormone (TSH) is the main regulator of thyroid proliferation and differentiation (Vassart, Dumont, 1992). It mediates its effect primarily through the activation of the cAMP cascade (Laglia *et al*., 1996). *NIS* expression is positively regulated via the cAMP pathway (Weiss *et al*., 1984). Research conducted for this thesis revealed a cAMP response element (CRE) in *THADA* (Kloth *et al*., 2011). Therefore, one might

speculate about a TSH controlled activation mechanism for *THADA* in the thyroid similar to the one described for *NIS*.

In addition to the thyroid, *NIS* is also expressed in salivary glands, gastric mucosa, and the lactating mammary gland (Bizhanova, Kopp, 2009). Nevertheless, it is considered a thyroid-specific gene (Vivaldi *et al*., 2009). This is not the case for *THADA*, NCBI ESTProfileViewer shows an almost ubiquitary expression of *THADA* in all tissues. For some of them the transcripts per million (TPM)-values are zero, but in these cases the EST pool is considerably smaller (average: 30,701 vs. 174,805 all other tissues; median: 20,430 vs. 122,252), possibly giving false negative results. Data gathered in this thesis verify those results in principle, even though some divergences could be detected. Foremost, in all samples an expression was noticed. NCBI ESTProfileViewer marks the thyroid as the tissue with the third highest expression in a group of 45 tissues. Only the pharynx and connective tissue have a higher TPM than detected in the thyroid. While testing eight different tissues, own data indicated a significantly higher expression in the thyroid than all other tissues (Kloth *et al*., 2011).

Overall, *THADA* expression seems to be relatively stable, in most studies undertaken as part of this thesis it showed a variance of no more than 1 to 14.95 times. This includes several cell lines, carcinomas of the lung, prostate carcinomas, and fetal placenta. In hematological and in thyroid lesions a range of 1 to 26.31 and 1 to 45.94, respectively, could be detected. This might be due to its yet unknown function or functions, but could also be related to its size. In its full-length (*-A1*) form, *THADA* has a genomic size of about 365 kbp, the cDNA of its transcript spans over 6.134 bp, distributed over 38 exons, and its protein has a predicted molecular weight of 220 kDa (GenBank accession number: NM_022065) (Rippe *et al*., 2003; Drieschner *et al*., 2007). Arranging all known human RNAs and proteins by size, the above numbers put THADA in the 91st (RNA) and 97th (protein) percentile, respectively (Rolf Nimzyk, personal communication; refseq database, without predicted RNAs/proteins, NCBI, September 2014). Therefore, a significant upregulation of the expression requires a large amount of cellular resources and transcription and translation take more time.

*THADA* variants have been associated with several diseases: type 2 diabetes (T2D), polycystic ovary syndrome (PCOS), nonsyndromic cleft lip (NSCL/P), multiple sclerosis (MS), and certain types of cancer (see also chapter 1). Ludwig *et al*. (2012) suggest that

this might be related to its large size, but that it could also reflect the effect of regulatory elements. Several single nucleotide polymorphisms (SNP) have been detected nearby *THADA* and in its genomic sequence. In the last couple of years most research on *THADA* focused on potential associations of *THADA* variants and certain diseases. In several studies no statistical significance could be reached. These sometimes conflicting results might be caused by ethnic specificity, but might also be due to an insufficiently sized cohort in relation to the allele frequency in some studies. Even though in some cases a connection between the diseases exists, generally they affect highly different tissues, possibly linking *THADA* to multiple cellular processes. This is illustrated by the results presented in Cheng *et al*. (2011), where a low risk T2D variant was found to be the high risk variant for colon cancer.

Interestingly, only one of the SNPs is located in the *THADA* ORF. rs7578597, the aforementioned SNP associated with T2D in its major allele variant and with colon cancer in its minor allel form, is located in a part of *THADA*, which turned out to be the most conserved region of its protein. Drieschner *et al*. (2007) were able to demonstrate that the region comprising of aa 1033 to 1415 in *Homo sapiens* has the highest similarity between five vertebrates. In the minor allel form rs7578597 represents a missense mutation, an aa with a polar functional group (threonine) becomes replaced by an aa with a hydrophobic side chain (alanine) in the protein. One might speculate that a possible transformation of the putative functional domain of the protein caused by this mutation could critically alter the biological effect of THADA. Green *et al*. (2010) observed a region of 336 kbp in *THADA* depleted of derived allels in Neanderthals. While citing Parikh *et al*. (2009; see chapter 4, page 3), the authors suggest that changes in *THADA* may have affected aspects of energy metabolism in early modern humans (Green *et al*., 2010). Cardona *et al*. (2014) marked *THADA* as a cold adaptation candidate gene. By genotyping of indigenous Siberian populations *THADA* was identified as a gene with unusually rapid allele frequency and long-range haplotype homozygosity change in the recent past (Cardona *et al*., 2014). The authors relate this change to a possible advantage in energy metabolism gained by mutations in *THADA*.

The conserved region reported by Drieschner *et al*. (2007) is also the target in thyroid adenomas when *THADA* becomes truncated. One of the studies undertaken for this thesis revealed that the 3'-part of the protein has no effect on the cellular location. Using pEGFP-

C1 and -N1 vectors and full-length (*THADA-A3*) and the truncated form of *THADA*, it could be shown that the protein is located in the cytoplasm of the cell, regardless of the existence of the 3'-part. Therefore, it is to be expected that the effect the truncated form of the protein exerts on the thyroid cell leading to the development of an adenoma does not involve a change of the localization of THADA. Drieschner *et al*. (2007) detected a homology to a protein-protein-interaction domain of the superfamily ARM-structure. Therefore, it seems more likely that a truncation of THADA might disrupt this putative protein-protein interaction. If THADA is indeed involved in the death receptor pathway as discussed by Rippe *et al*. (2003), and Drieschner *et al*. (2007), this might explain the development of adenomas with a 2p21 rearrangement.

The second main aspect of this thesis was the quantitative analysis of *high-mobility group AT-hook 2* (*HMGA2*) in dedifferentiated and extra-embryonic human tissues. Re-expression of *HMGA2* is often found in malignant tumors (reviewed in Cleynen, Van de Ven, 2008; Fedele, Fusco, 2010). An upregulation in *HMGA2* expression has been associated with a progressive dedifferentiation of thyroid tumors (Belge *et al*., 2008). The expression level of *HMGA2* turned out to be negatively associated with the one of *THADA* in the thyroid (Kloth *et al*., 2011). In conjunction with the positive correlation with *NIS*, this finding further verifies *THADA* as a marker for the dedifferentiation of thyroid epithelial cells. In other tissues this correlation could not be reproduced. Neither in samples of hematologic diseases (ALL, CLL, AML, CML, other myeloproliferative neoplasm, malignant lymphoma), in carcinomas of the lung (adenocarcinoma, squamous cell carcinoma) and the prostate, nor in fetal placenta specimens a significant correlation between *THADA* and *HMGA2* could be detected. In thyroid cell lines results hinted at a trend towards a positive correlation. Common to cell culture, examined cell lines derived from benign tumors had been immortalized and it is to be expected that *in vitro* conditions led to further changes of the cells in all cases. Therefore, a correlation with the tumor the cell line originated from might not be given anymore. In no instance *THADA* expression showed a significant difference between any of the clinical subgroups. Additionally, when comparing *THADA* expression in lesions versus normal samples of the same type of tissue, no significant differences could be detected. This could indicate that *THADA* was not involved in the degeneration of the cells in these tissues. Taken together, results might

suggest that the biological effect of *THADA* in thyroid cells is different from other tissues. At the current state of knowledge, with the exception of ß-cells in correlation with T2D as reported by Voight *et al*. (2010), the correlation of the biological effects of *THADA* and *HMGA2* seems to be restricted to the thyroid.

Whereas only relatively small differences in expression level of *THADA* could be detected outside the thyroid, considerable variations in *HMGA2* expression were ascertained in extra-embryonic and in dedifferentiated tissues. In fetal placenta an investigation comprised of 90 samples revealed a highly significant correlation between the expression value and the calendar gestational age (CGA) (Kloth *et al*., in preparation). During the first trimester, while the uterus is a low oxygen environment, high *HMGA2* expression was detected. During this period the proliferation of the placenta is higher than the one of the fetus. This changes later with an apparent cross-over around mid-gestation (Sitras *et al*., 2012). For the avoidance of several pregnancy-related complications like placenta accreta, increta, and percreta, and preeclampsia, a correct implantation of the embryo into the uterus is crucial. In a process similar to cancerous growth and invasion, the fetal trophoblast invades the maternal decidua. Normally, this happens in a spatially and temporally tightly restricted manner. In case of placenta accreta, increta, and percreta chorionic vili can invade into or even through the myometrium (Belfort, 2010). In preeclampsia, cytotrophoblast differentiation is abnormal and invasion into the decidualized endometrium is shallow (Genbacev *et al*., 1996). While the syncytiotrophoblast remains mostly epithelial, the extravillous trophoblast (EVT) goes through the process of epithelial-mesenchymal transition (EMT) (Vićovac, Aplin, 1996). Own research showed nuclear HMGA2 in stromal cells of the placental villi, and in case of a true signal, cytoplasmatic HMGA2 in the trophoblast (Kloth *et al*., in preparation). Therefore, *HMGA2* might not to be involved in the EMT of the EVT cells in the placenta. Bamberger *et al*. (2003) reported the detection of HMGA1 in the EVTs. They noticed a switch from nuclear to cytoplasmatic expression of *HMGA1* when cytotrophoblast cells differentiate into EVTs. Final determination if this switch also happens for *HMGA2* might be achieved by separately testing stroma and trophoblast by qRT-PCR. The necessary separation of the cell layers could be performed by laser dissection. Cytoplasmatic expression of *HMGA2* has been reported only rarely (Genbacev *et al*., 2011; Ding *et al*.,

2014). The authors do not give a possible explanation as to the role of HMGA2 in cytoplasm. For *HMGA1*, a role similar to *HMGB1* has been discussed (Bamberger *et al*., 2003). In certain cell types, HMGB1 can be secreted and after retaining its association with the plasma membrane, regulate cell migration (Fages *et al*., 2000). Bamberger *et al*. (2003) also address the ability of HMGB1 to activate extracellular proteases, which are of importance for tissue invasion. Although no direct connection between *HMGA2* and the above mentioned obstetric complications could be detected, future research might advance the knowledge on that subject using the present study as groundwork.

To elucidate if the expression of *HMGA2* might serve as a marker to distinguish between the different types of hydatidiform moles, non-molar hydropic abortions, and normal pregnancies, eight such samples were tested. Hydatidiform moles are characterized by a specific genetic setting. CHMs are usually diploid and, as first reported by Kaji and Ohama (1977), of androgenetic origin. In the majority of cases partial moles are triploid and diandric (reviewed in Hoffner, Surti, 2012). These genetic characteristics are used in ancillary techniques to support the pathological assessment (Kipp *et al.*, 2010). Differences between final results and initial diagnoses are not uncommon (Niemann *et al.*, 2007; Kipp *et al.*, 2010; Sarmadi *et al.*, 2011). The results of the present study support the findings by Kipp *et al.* (2010). The macro- and microscopical determination was verified by immunostaining with a $p57^{KIP2}$-specific antibody and the determination of the ploidy by FISH. After consultation with the pathologist, four of the samples were ascertained as complete moles. Contrary to the initial diagnosis of partial moles, two of the four samples could be identified as hydropic abortions, one was determined as a partial mole with a tetraploid chromosome set. Kipp *et al*. (2010) presented one case with identical FISH and $p57^{KIP2}$ results, but because of the pathological analysis they favored a hydropic abortion as the final diagnosis. Tetraploid PHMs are rare but have been described before (Surti *et al*., 1986; Vejerslev *et al*., 1987; Lawler *et al*., 1991). These results underline the necessity of ancillary techniques to correctly identify hydatidiform moles and non-molar hydropic abortions and adapt the therapy accordingly.

Briese *et al*. (2006) investigated CHMs for the expression of HMGA1 and found it to be similar to that of normal placenta tissue. Own results indicate that the same is true for HMGA2. A less intensive staining in comparison to the non-molar placenta samples at the same gestational age, where the latter was available, might hint at a difference, but a

larger sample size would be necessary for clarification.

Differences in the expression level of *HMGA2* have been detected between normal tissue and benign and malignant neoplasms in a multitude of cases (reviewed in Fedele, Fusco, 2010). For a final evaluation a larger set of samples is necessary, but as a preliminary result, the expression level of *HMGA2* is not a marker to distinguish normal placentas from hydatidiform moles and non-molar hydropic abortions nor to distinguish between CHMs and PHMs.

Utilizing the highly sensitive method of real-time PCR in combination with immunohistochemistry, *HMGA2* expression was investigated on 59 samples of post-pubertal testicular germ-cell tumors (TGCT). Previous studies relied on the less sensitive RT-PCR method (Franco *et al*., 2008) or a very small sample pool size of three specimens (two of which were pediatric) (Murray *et al*., 2013). Opposite to the study by Franco *et al*., (2008), a baseline expression could be detected in all but one case. This is probably due to the higher sensitivity of the qRT-PCR and might be caused by the normal tissue percentage of each sample. Statistical analysis revealed a separation of seminomas from all other samples by qRT-PCR alone with a high sensitivity and a moderately high specificity. Combining the results from the qRT-PCR with the data gathered after the immunohistochemical investigation, a highly significant difference between the YSTs and all other specimens could be detected. Different molecular markers like OCT3/4, c-KIT, NANOG, and SOX2, amongst others, have been used to determine the pathogenetic progression of this heterogeneous group of neoplasias (Honecker *et al*., 2006; Gopalan *et al*., 2009). The widely accepted model marks the “intratubular germ cell neoplasia undifferentiated” (ITGCNU) as the initial lesion (Oosterhuis, Looijenga, 2005; Honecker *et al*., 2006; Franco *et al*., 2008; Gopalan *et al*., 2009; Chieffi, 2011; Chieffi, Chieffi, 2013). Except for rare cases of “burned out” ITGCNUs, in all cases eventually a testicular germ cell tumor develops (Hoei-Hansen *et al.,* 2005). This can be a seminoma or an EC. It is believed that through reprogramming a seminoma can be transformed into an EC, but not an EC into a seminoma (Oosterhuis, Looijenga, 2005; Gopalan *et al*., 2009; Chieffi, 2011; Chieffi, Chieffi, 2013). An EC can further progress into a teratoma, a YST, or a CC (Oosterhuis, Looijenga, 2005; Honecker *et al*., 2006; Gopalan *et al*., 2009; Chieffi, 2011; Chieffi, Chieffi, 2013). This model is supported by the data from the present study. *HMGA2* expression was very low to low in seminomas and ECs, and overall moderate to high in

teratomas, YSTs and CCs. Therefore, the activation of *HMGA2* seems to take place when ECs progress into one of the other non-seminomas. Some markers used for the above model are also utilized in histopathological evaluation of TGCTs, in addition to others. Despite these, false diagnoses up to 32 % have been reported (Segelov *et al*., 1993; Lee *et al*., 1999; Delaney *et al*., 2005). Results indicate that an HMGA2-specific antibody might be a reasonable addition to the set, possibly reducing the risk of a false diagnosis. Since treatment and follow-up depends on correct determination, future research should further advance this subject to bring it to clinical application.

In summary, first insights about the role of *THADA* in dedifferentiated thyroid tumors were revealed. Additionally, the cytoplasmatic localization of its protein was determined. *THADA* and *HMGA2* have been investigated rarely together. Research undertaken as part of this thesis elucidated a negative correlation between the expression of both genes, detected in thyroid neoplasias. Further studies could not find this correlation in other tissues, making it exclusive to the thyroid. Together with the high expression of *THADA* in this organ these results further emphasize its role in the thyroid. High expression of *HMGA2* in fetal placenta samples of low gestational age reaffirms the role in early development. An expression pattern deviating from the one of *HMGA1* as reported by Briese *et al*. (2006) suggests a different role of the two *HMGA* genes in this tissue. In TGCTs a tumor subtype-specific expression could be detected. Results strongly suggest HMGA2 as a promising marker in the pathological determination of samples in clinical application.

# 5. Summary

At the onset of this thesis very little was known about *THADA* (*thyroid adenoma associated*). The gene had been discovered when a breakpoint on chromosome 2 commonly found in thyroid adenomas was narrowed down to a locus in band p21. Analysis of the genomic structure of *THADA* revealed a size of 365 kbp and 38 exons in the full-length form. In case of the aforementioned truncation the breakpoints are located in intron 28, thereby translocating the 3'-end of the gene. Own research elucidated that its protein is located in the cytoplasm, independent of the existence of the carboxy terminus encoded by exons 29 to 38. Therefore, the development of a thyroid adenoma caused by the truncation of *THADA* does not seem to coincide with a change of the location of its protein.

Besides benign adenomas, there are four main types of malignant neoplasias of the thyroid. The usually relatively differentiated papillary and follicular types of cancer are associated with a better prognosis, the less differentiated medullary and anaplastic subtypes with a lower survival rate of the patients. *THADA* turned out to be a marker of dedifferentiation of thyroid tissue. One of the cellular processes *THADA* may be involved in is maintaining the differentiation of the follicular epithelium. In the thyroid *THADA* might have a particular role, since its expression turned out to be significantly higher in this organ than in several other tissues, determined by real-time PCR. Also, only in the thyroid a negative correlation with *HMGA2* could be detected in a study analyzing the expression of both genes on neoplastic tissue samples, cell lines and fetal placenta specimens. *HMGA2* is a marker for the dedifferentiation of thyroid tissue, thereby verifying the results obtained in the analysis of *THADA* expression.

*HMGA2* is known to play an important role in early development. This could be confirmed for fetal placenta samples. High qRT-PCR values were detected in samples from the first trimester, whereas a baseline expression could be observed up until birth. A protein expression pattern diverging from the one reported for HMGA1 hints at different roles for both proteins. Hydatidiform moles are a pathological type of pregnancy. They are characterized by a usually triploid, diandric (partial hydatidiform mole), or a diploid, paternal (complete hydatidiform mole) chromosome set. Since it is of clinical relevance to distinguish between the subtypes, a marker would be of high interest. Due to a limited

sample size, no final conclusion could be drawn. Results indicate no significant difference in expression between the subtypes and between these and normal placenta specimens, excluding *HMGA2* as a marker.

While *HMGA2* is mostly downregulated in adult tissues, reexpression can be found in several tumors, especially in malignant neoplasias. This is also the case for testicular germ cell tumors. These consist of seminomas, embryonal carcinomas, yolk sac tumors, teratomas, and choriocarcinomas. Studies preceding own investigations reported an expression level depending on the subgroup, but used the less sensitive RT-PCR or a very small sample size. The subgroup-specific expression could not only be more distinctively determined, but it could also be shown, that use of qRT-PCR and particularly immunohistochemistry on HMGA2 might serve as a marker in clinical application.

# 6. Zusammenfassung

Zu Anfang dieser Doktorarbeit war sehr wenig über *THADA* (*thyroid adenoma associated*) bekannt. Das Gen war entdeckt worden, als ein in Schilddrüsenadenomen häufig anzutreffender Bruchpunkt in Chromosom 2 auf einen Genlokus in Bande p21 eingegrenzt werden konnte. Eine Analyse der genomischen Struktur von *THADA* ergab eine Größe von 365 kbp und 38 Exons bei der vollständigen Form des Gens. Im Falle der erwähnten Trunkierung ist Intron 28 betroffen, wobei das 3'-Ende des Gens transloziert wird. Eigene Ergebnisse ergaben eine Lokalisierung des Proteins im Cytoplasma, unabhängig von der Existenz des carboxyterminalen Endes, welches von den Exons 29 bis 38 kodiert wird. Daher ist anzunehmen, dass es bei der Trunkierung von *THADA*, welche zu einer Entstehung eines Adenoms führt, nicht zu einer Änderung der Lokalisation des Proteins kommt.

Neben den gutartigen Adenomen gibt es vier Hauptgruppen an malignen Neoplasien der Schilddrüse. Die üblicherweise relativ differenzierten papillären und follikulären Formen beinhalten eine bessere Prognose, die weniger differenzierten medullären und anaplastischen Formen bedeuten eine geringere Überlebensrate für den Patienten. Es stellte sich heraus, dass *THADA* ein Marker der Dedifferenzierung von Schilddrüsengewebe ist. Einer der zellulären Prozesse, in die *THADA* involviert sein könnte, ist die Aufrechterhaltung der Differenzierung des follikulären Epithels. In der Schilddrüse könnte *THADA* eine besondere Rolle spielen, da sich in diesem Organ die Genexpression als signifikant höher als in mehreren anderen Geweben herausstellte. Dazu wurde nur in der Schilddrüse eine negative Korrelation mit *HMGA2* als Teil einer Studie detektiert, bei der die Expression von beiden Genen in neoplastischen Gewebeproben, Zelllinien und fetalen Plazenta-Proben gemessen wurde. *HMGA2* ist ein Marker der Dedifferenzierung von Schilddrüsengewebe, wodurch die Ergebnisse, welche bei der Untersuchung an *THADA* gewonnen werden konnten, verifiziert wurden.

Von *HMGA2* ist bekannt, dass es eine wichtige Rolle in der Frühentwicklung spielt. Dieses konnte für fetale Plazenta-Proben bestätigt werden. Hohe qRT-PCR-Werte wurden in Proben aus dem ersten Trimester gefunden, wohingegen im weiteren Schwangerschaftsverlauf bis zur Geburt eine Grundexpression feststellbar war. Eine von der berichteten Protein-Expression von HMGA1 abweichende Expression deutet auf

unterschiedliche Rollen der beiden Proteine hin. Blasenmolen stellen eine pathologische Form der Schwangerschaft dar. Sie sind durch einen typischerweise triploiden, diandrischen (partielle Blasenmole), oder durch einen diploiden, paternalen (vollständige Blasenmole) Chromosomensatz gekennzeichnet. Da es von klinischer Relevanz ist zwischen den Subtypen unterscheiden zu können, wäre ein Marker von großem Interesse. Aufgrund eines begrenzten Probensatzes konnte keine endgültige Schlussfolgerung gezogen werden. Die Ergebnisse deuten auf keinen signifikanten Unterschied der Expression zwischen den Subtypen und zwischen diesen und normalen Plazenta-Proben hin, welches *HMGA2* als Marker ausschließt.

Während *HMGA2* in adulten Geweben weitestgehend herunter reguliert ist, kann in verschiedenen Tumoren eine Reexpression gefunden werden. Dies ist auch bei testikulären Keimzelltumoren der Fall. Diese setzen sich aus Seminomen, Embryonalkarzinomen, Dottersacktumoren und Chorionkarzinomen zusammen. Studien, die eigenen Untersuchungen voraus gingen, berichteten von einer Abhängigkeit des Expressionsniveaus vom Subtyp, verwendeten allerdings die weniger sensitive RT-PCR oder einen sehr kleinen Probenumfang. Es konnte nicht nur die Subgruppen-spezifische Expression in deutlicherem Maße bestimmt werden, sondern auch gezeigt werden, dass qRT-PCR und insbesondere Immunhistochemie an HMGA2 als Marker in der klinischen Anwendung Einsatz finden könnte.

# 7. List of publications

## 7.1. Oral presentations

"Quantifizierung der Expression des *THADA*-Gens in soliden Tumoren"
24. Treffen der Norddeutschen Humangenetiker, Kiel, Germany, 12.11.2005

"*THADA*-Expression in verschiedenen Schilddrüsen-Läsionen"
30. Treffen der Norddeutschen Humangenetiker, Kiel, Germany, 05.11.2011

## 7.2. Poster presentations

(I) "THADA - a protein with an important role in the thyroid"
55. Symposium der Deutschen Gesellschaft für Endokrinologie, Mannheim, Germany, 07.03.-10.03.2012

## 7.3. Peer-reviewed papers

(II) Kloth L, Belge G, Burchardt K, Loeschke S, Wosniok W, Fu X, Nimzyk R, Mohamed SA, Drieschner N, Rippe V, Bullerdiek J. Decrease in thyroid adenoma associated (*THADA*) expression is a marker of dedifferentiation of thyroid tissue. BMC Clin Pathol 2011, 11:13.

(III) Kloth L, Helmke BM, Wosniok W, Drieschner N, Belge G, Burchardt K, Bullerdiek J. Expression of *HMGA2* in fetal placenta correlates with gestational age. (in preparation)

(IV) Kloth L, Gottlieb A, Helmke BM, Wosniok W, Löning T, Belge G, Günther K, Bullerdiek J. HMGA2 expression distinguishes between different types of post-pubertal testicular germ cell tumours. J Pathol: Clin Res 2015. (accepted)

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# 10. Appendix

The following tables 4-8 contain the expression data for *THADA* and *HMGA2* used to determine if a suspected correlation between the two genes exists in tissues other than the thyroid (see section 3.3).

**Table 4:** *THADA* and *HMGA2* expression in cell cultures.

| sample | *THADA* RQ | *HMGA2* RQ | tissue |
|---|---|---|---|
| S40.2/TSV40 | 5.463 | 236.123 | thyroid adenoma |
| S121/TSV40 | 2.582 | n.a. | thyroid adenoma |
| S141.2/TSV40 | 5.130 | n.a. | thyroid adenoma |
| S211/TSV40 | 3.722 | 194.444 | thyroid adenoma |
| S325/TSV40 | 3.976 | n.a. | thyroid adenoma |
| S533/TSV40 | 3.106 | n.a. | thyroid adenoma |
| FTC133 | 5.994 | 160.990 | follicular thyroid carcinoma |
| FTC238 | 6.054 | 185.396 | follicular thyroid carcinoma |
| HTH74 | 7.079 | n.a. | anaplastic thyroid carcinoma |
| S277 | 14.950 | 270.937 | anaplastic thyroid carcinoma |
| Jurkat | 1.000 | 0.010 | T-cell leukemia |
| SupT1 | 6.160 | 475.736 | T-cell leukemia |
| Myco 1T | 8.615 | 0.629 | pleomorphic adenoma of the salivary glands |
| Li14/TSV40 | 3.301 | 237.012 | lipoma |
| Fi primary cells | 3.536 | n.a. | skin fibroblasts |

*HMGA2* expression data was kindly provided by Anke Meyer (Center for Human Genetics, University of Bremen). RQ: relative quantification; tissue: tissue (tumor type) cell line deriving from; n.a.: not available.

**Table 5:** *THADA* and *HMGA2* expression in carcinomas of the lung.

| sample | type | histology | age | grade | stage | *THADA* RQ | *HMGA2* AQ |
|---|---|---|---|---|---|---|---|
| A1 | cancer | ac | 61 | G1 | 1B | 2.072 | 385,069 |
| A1n | normal | | | | | 1.689 | 7,519 |
| A2 | cancer | ac | 73 | G3 | 3A | 2.579 | 7,153 |
| A2n | normal | | | | | 2.283 | 289 |
| A3 | cancer | ac | 70 | G3 | 3A | 4.806 | 8,320,000 |
| A3n | normal | | | | | 1.000 | 525,947 |

| sample | type | histology | age | grade | stage | *THADA* RQ | *HMGA2* AQ |
|---|---|---|---|---|---|---|---|
| A4 | cancer | ac | 62 | G2 | 2B | 1.528 | 616,236 |
| A4n | normal | | | | | 2.742 | 69,511 |
| A7 | cancer | ac | 63 | G3 | 3A | 1.489 | 5,422 |
| A7n | normal | | | | | 1.750 | 1,664 |
| A8 | cancer | ac | n.a. | G2 | n.a. | 4.394 | 123,801 |
| A8n | normal | | | | | 3.174 | 8,203 |
| A9 | cancer | ac | 57 | G3 | 2A | 2.723 | 127,197 |
| A9n | normal | | | | | 2.633 | 2,331 |
| A10 | cancer | ac | 61 | G2 | 4 | 2.338 | 16,470 |
| A10n | normal | | | | | 1.830 | 2,542 |
| A11 | cancer | ac | 61 | G3 | 3A | 1.721 | 5,584,977 |
| A11n | normal | | | | | 4.813 | 192,350 |
| P1 | cancer | sc | 70 | G2 | 2B | 3.151 | 3,528,705 |
| P1n | normal | | | | | 1.462 | 337,270 |
| P2 | cancer | sc | n.a. | G2 | 2B | 5.165 | 7,778,535 |
| P2n | normal | | | | | 2.395 | 8,954 |
| P3 | cancer | sc | 64 | G3 | 1A | 1.472 | 362,274 |
| P3n | normal | | | | | 2.426 | 8,611 |
| P4 | cancer | sc | 62 | G1 | 3B | 4.182 | 13,749,450 |
| P4n | normal | | | | | 2.285 | 55,266 |
| P5 | cancer | sc | 62 | G2 | 2B | 2.126 | 164,355 |
| P5n | normal | | | | | 2.619 | 15,855 |
| P6 | cancer | sc | 74 | G1 | 3B | 1.725 | 1,321,792 |
| P6n | normal | | | | | 2.212 | 11,754 |
| P7 | cancer | sc | 69 | G1 | 1B | 3.663 | 11,478,720 |
| P7n | normal | | | | | 1.989 | 14,884 |
| P8 | cancer | sc | 62 | G3 | 2B | 2.749 | 31,432 |
| P8n | normal | | | | | 1.915 | 5,802 |
| P9 | cancer | sc | 59 | G1 | 2B | 2.445 | 1,243,528 |
| P9n | normal | | | | | 1.910 | 4,423 |

*HMGA2* expression data (absolute quantification) was kindly provided by Britta Meyer (Center for Human Genetics, University of Bremen). Type: type of tissue, cancer or corresponding normal tissue; RQ: relative quantification; AQ: absolute quantification, number of transcripts/250 ng total RNA; n.a.: not available.

# Appendix

**Table 6:** *THADA* and *HMGA2* expression in prostate carcinomas.

| sample | Gleason grading | Gleason score | *THADA* RQ | *HMGA2* RQ |
|---|---|---|---|---|
| P01 | 3+3 | 6 | 1 | 6.01 |
| P02 | 2+3 | 5 | 1.142 | 4.95 |
| P03 | 4+3 | 7 | 1.057 | 4.41 |
| P04 | 4+3 | 7 | 1.309 | 1.00 |
| P05 | 5+4 | 9 | 3.047 | 20.69 |
| P06 | 4+4 | 8 | 2.048 | 3.56 |
| P07 | 4+4 | 8 | 1.122 | 290.11 |
| P08 | 3+3 | 6 | 2.805 | n.a. |
| P09 | 3+3 | 6 | 2.119 | 7.04 |
| P10 | 5+4 | 9 | 1.112 | 4.03 |
| P11 | 4+5 | 9 | 1.383 | 148.71 |
| P12 | 3+3 | 6 | 2.205 | 7.61 |
| P13 | 4+4 | 8 | 1.412 | 5.61 |
| P14 | 4+3 | 7 | 3.312 | 16.50 |
| P15 | 4+4 | 8 | 1.664 | 41.61 |

*HMGA2* expression data was kindly provided by Anke Meyer (Center for Human Genetics, University of Bremen). RQ: relative quantification; n.a.: not available.

**Table 7:** *THADA* and *HMGA2* expression in hematologic diseases including outside controls.

| sample | *THADA* RQ | *HMGA2* AQ | classification | therapy | age | cell no. |
|---|---|---|---|---|---|---|
| 30 | 1.533 | n.a. | normal blood | - | n.a. | n.a. |
| 31 | 0.703 | n.a. | normal blood | - | n.a. | n.a. |
| 32 | 1.232 | n.a. | normal blood | - | n.a. | n.a. |
| 33 | 0.904 | n.a. | normal blood | - | n.a. | n.a. |
| 34 | 0.860 | n.a. | normal blood | - | n.a. | n.a. |
| 35 | 0.942 | n.a. | normal blood | - | n.a. | n.a. |
| 36 | 0.826 | n.a. | normal blood | - | n.a. | n.a. |
| B03 | 1.287 | 16,431 | CLL | 1 | 59 | 30.9 |
| B18 | 2.101 | 4,298 | CLL | 1 | 65 | 13.2 |
| B54 | 0.765 | 7,112 | CLL | 2 | 57 | 5 |
| B32 | 0.636 | 388,139 | ALL | 0 | 39 | 4.8 |

| sample | *THADA* RQ | *HMGA2* AQ | classification | therapy | age | cell no. |
|---|---|---|---|---|---|---|
| B01 | 0.899 | 13,583 | ALL | 1 | 29 | 11.1 |
| B07 | 0.604 | 13,583 | ALL | 1 | 29 | 9.2 |
| B06 | 1.847 | 10,937 | ALL | 2 | 44 | 5.8 |
| B17 | 0.134 | 36,873 | ALL | 0 | 61 | 2.9 |
| B35 | 0.387 | 94,313 | ALL | 1 | 67 | 2.5 |
| B08 | 0.411 | 21,306 | ALL | 2 | 34 | 7 |
| B25 | 1.052 | 6,037 | CML | 1 | 68 | 5.5 |
| B05 | 0.549 | 29,062 | CML | 1 | 61 | 8.9 |
| B14 | 1.109 | 49,718 | CML | 1 | 66 | 13.1 |
| B57 | 0.948 | 12,949 | CML | 1 | 68 | 6.5 |
| B60 | 0.369 | 7,918 | CML | 1 | 54 | 3 |
| B22 | 2.250 | n.a. | CML | 1 | 64 | 40 |
| B33 | 1.034 | 2,686,810 | AML | 0 | 67 | 1.2 |
| B28 | 0.673 | 9,045 | AML | 0 | 96 | 10 |
| B62 | 0.639 | 6,708 | AML | 2 | 51 | 8 |
| B26 | 1.810 | 387,910 | AML | 0 | 68 | 9.6 |
| B15 | 0.483 | 11,462 | AML | 1 | 66 | 5.8 |
| B29 | 0.336 | 9,619 | AML | 0 | 69 | 2.2 |
| B36 | 1.101 | 7,887 | AML | 0 | 44 | 17 |
| B53 | 0.281 | 11,242 | AML | 0 | 74 | 1.3 |
| B04 | 1.374 | 15,297 | AML | 2 | 56 | 5.9 |
| B37 | 0.430 | 9,331 | AML | 2 | 68 | 6.2 |
| B34 | 0.362 | 84,906 | AML | 0 | 80 | 2.9 |
| B31 | 1.968 | 2,228,310 | AML | 0 | 71 | 1.8 |
| B16 | 0.551 | 53,298 | AML | 0 | 69 | 1.4 |
| B52 | 2.955 | 598,278 | AML | 1 | 85 | 70 |
| B21 | 3.512 | 3,936 | AML | 1 | 84 | 2.2 |
| B13 | 0.660 | 8,604 | other myeloproliferative neoplasm | 1 | 70 | 4.5 |
| B24 | 0.373 | 9,423 | other myeloproliferative neoplasm | 0 | 72 | 2.4 |
| B58 | 0.642 | 43,743 | other myeloproliferative neoplasm | 1 | 75 | 76.1 |
| B66 | 0.997 | 3,399 | other myeloproliferative neoplasm | 1 | 57 | 3.4 |
| B68 | 0.755 | 10,150 | other myeloproliferative neoplasm | 1 | 74 | 4.1 |
| B46 | 1.251 | 5,396 | other myeloproliferative neoplasm | 1 | 61 | 3.8 |
| B65 | 0.435 | 18,359 | other myeloproliferative neoplasm | 1 | 48 | 3 |
| B50 | 0.871 | 5,369 | other myeloproliferative neoplasm | 0 | 57 | 11.5 |
| B02 | 0.456 | 10,492 | malignant lymphoma | 1 | 66 | 1.7 |
| B20 | 1.710 | 605,254 | malignant lymphoma | 2 | 57 | 4.6 |
| B23 | 0.999 | 2,767 | malignant lymphoma | 2 | 57 | 5.1 |

# Appendix

| sample | *THADA* RQ | *HMGA2* AQ | classification | therapy | age | cell no. |
|---|---|---|---|---|---|---|
| B40 | 1.159 | 1,451 | malignant lymphoma | 2 | 57 | n.a. |
| B49 | 0.462 | 7,236 | malignant lymphoma | 2 | 62 | 3.7 |
| B43 | 0.673 | 10,632 | malignant lymphoma | 0 | 68 | 6.1 |
| B47 | 0.989 | 6,821 | malignant lymphoma | 0 | 83 | 9.8 |
| B64 | 0.815 | 2,870 | malignant lymphoma | 2 | 42 | 9.1 |
| B41 | 1.736 | 4,444 | malignant lymphoma | 0 | 24 | 12.4 |
| B42 | 1.321 | 7,712 | malignant lymphoma | n.a. | 37 | n.a. |
| B45 | 0.522 | 6,291 | other neoplasia | 1 | 68 | 8 |
| B67 | 1.104 | 4,123 | other neoplasia | 0 | 48 | 6.2 |
| B44 | 0.802 | 5,469 | systemic lupus erythematosus | 1 | 58 | 8.3 |
| B59 | 0.714 | 9,476 | systemic lupus erythematosus | 1 | 59 | 4 |
| B55 | 1.401 | 4,737 | Takayasu syndrome | 1 | 34 | 7.2 |
| B61 | 0.487 | 6,226 | dermatomyositis | 1 | 66 | 4.2 |
| B63 | 1.169 | 6,878 | amyloidosis of the kidney | 1 | 65 | 6.4 |

*HMGA2* expression data (absolute quantification) was kindly provided by Britta Meyer (Center for Human Genetics, University of Bremen). RQ: relative quantification; AQ: absolute quantification, number of transcripts/250 ng total RNA; therapy: phase of therapy, blood sample taken 0: before, 1: during: 2: after therapy; cell no.: cell number in Giga/l; n.a.: not available.

**Table 8:** *THADA* and *HMGA2* expression in fetal placenta.

| sample | *THADA* RQ | *HMGA2* RQ | classification | CGA |
|---|---|---|---|---|
| P001 | 11.673 | 140.523 | SA | 7 |
| P002 | 4.681 | 9.902 | SA | 7 |
| P003 | 3.106 | 3.120 | AR | 15 |
| P004 | 4.480 | 95.469 | SA | 9 |
| P005 | 9.035 | 46.382 | SA | 10 |
| P006 | 4.697 | 68.581 | SA | 8 |
| P007 | 3.602 | 36.058 | SA | 8 |
| P008 | 3.756 | 32.201 | AR | 8 |
| P009 | 6.744 | 29.015 | SA | 8 |
| P010 | 8.260 | 497.846 | SA | 8 |
| P011 | 3.386 | 19.692 | SA | 10 |
| P012 | 4.787 | 89.261 | SA | 9 |
| P013 | 4.461 | 67.173 | AR | 6 |
| P014 | 7.197 | 99.902 | SA | 9 |
| P015 | 8.130 | 19.433 | SA | 14 |

| sample | *THADA* RQ | *HMGA2* RQ | classification | CGA |
|---|---|---|---|---|
| P016 | 6.236 | 45.969 | SA | 10 |
| P017 | 4.831 | 49.632 | n.a. | 7 |
| P018 | 5.614 | 141.657 | SA | 10 |
| P019 | 3.626 | 4.725 | SA | 11 |
| P020 | 4.787 | 13.690 | SA | 9 |
| P021 | 5.945 | 3.507 | SA | 28 |
| P022 | 7.689 | 48.989 | SA | n.a. |
| P023 | 7.386 | 130.567 | SA | 7 |
| P024 | 7.811 | 109.029 | AR | 8 |
| P025 | 7.760 | 188.477 | AR | 7 |
| P026 | 7.232 | 9.020 | SA | 10 |
| P027 | 5.717 | 85.272 | SA | 11 |
| P028 | 5.697 | 55.483 | SA | 8 |
| P029 | 4.587 | 136.494 | AR | 7 |
| P030 | 6.689 | 32.358 | AR | 8 |
| P031 | 10.319 | 96.846 | SA | 8 |
| P032 | 8.720 | 64.117 | SA | 7 |
| P033 | 3.949 | 1.027 | SA | 18 |
| P034 | 10.295 | 23.595 | SA | 22 |
| P035 | 5.551 | 68.913 | AR | 8 |
| P036 | 4.295 | 244.977 | SA | 8 |
| P037 | 6.748 | 23.640 | SA | 10 |
| P038 | 6.969 | 39.999 | SA | 11 |
| P039 | 5.709 | 24.045 | SA | 9 |
| P040 | 5.051 | 2.220 | AR | 20 |
| P041 | 11.484 | 1.057 | SA | 32 |
| P042 | 4.815 | 198.117 | SA | 9 |
| P043 | 9.984 | 126.931 | n.a. | n.a. |
| P044 | 7.177 | 87.735 | AR | 7 |
| P045 | 7.469 | 26.091 | SA | 8 |
| P046 | 5.248 | 8.117 | SA | 9 |
| P047 | 7.331 | 54.529 | SA | 9 |
| P048 | 8.654 | 30.706 | SA | 9 |
| P049 | 3.626 | 9.355 | SA | 7 |
| P050 | 5.280 | 80.126 | SA | 8 |
| P051 | 7.949 | 21.238 | SA | 10 |
| P052 | 5.354 | 44.867 | SA | 9 |
| P053 | 3.937 | 1.000 | SA | 7 |

# Appendix

| sample | *THADA* RQ | *HMGA2* RQ | classification | CGA |
|---|---|---|---|---|
| P054 | 8.890 | 123.155 | AR | n.a. |
| P055 | 4.059 | 152.952 | SA | 19 |
| P056 | 6.165 | 8.690 | AR | n.a. |
| P057 | 6.976 | 27.170 | n.a. | n.a. |
| P058 | 5.886 | 63.482 | SA | 7 |
| P059 | 8.346 | 250.892 | SA | 11 |
| P060 | 5.476 | 15.773 | AR | 7 |
| P061 | 13.142 | 160.201 | SA | 7 |
| P062 | 10.787 | 247.132 | SA | 11 |
| P063 | 10.366 | 92.824 | SA | 7 |
| P064 | 9.752 | 74.893 | SA | 9 |
| P065 | 5.953 | 162.155 | SA | 7 |
| P066 | 5.886 | 126.681 | AR | 6 |
| P067 | 7.799 | 186.271 | SA | 9 |
| P068 | 4.902 | 28.572 | AR | 8 |
| P069 | 5.264 | 47.861 | AR | 7 |
| P070 | 7.583 | 31.963 | SA | 9 |
| P071 | 7.461 | 154.653 | SA | 7 |
| P072 | 6.169 | 95.824 | AR | 7 |
| P073 | 5.646 | 31.415 | AR | 9 |
| P074 | 6.437 | 72.805 | SA | 7 |
| P075 | 8.984 | 56.650 | SA | 10 |
| P076 | 1.000 | 110.908 | SA | 20 |
| P077 | 5.626 | 6.058 | SA | 7 |
| P078 | 8.197 | 22.586 | SA | 14 |
| P079 | 9.102 | 179.214 | SA | n.a. |
| P080 | 6.697 | 123.656 | AR | 7 |
| P081 | 4.252 | 28.452 | SA | 9 |
| P082 | 3.465 | 18.370 | AR | 5 |
| P083 | 5.228 | 20.153 | AR | 7 |
| P084 | 3.039 | 87.926 | SA | 8 |
| P085 | 7.417 | 109.971 | SA | 12 |
| P086 | 5.394 | 152.111 | SA | 5 |
| P087 | 8.787 | 128.672 | SA | 7 |
| P088 | 6.240 | 47.139 | SA | 9 |
| P089 | 7.433 | 1.782 | n.a. | n.a. |
| P090 | 9.606 | 3.306 | n.a. | 28 |
| P091 | 13.047 | 1.865 | n.a. | n.a. |

| sample | *THADA* RQ | *HMGA2* RQ | classification | CGA |
|---|---|---|---|---|
| P092 | 3.453 | 4.502 | n.a. | 30 |
| P093 | 7.724 | 23.446 | SA | 27 |
| P094 | 6.823 | 6.276 | SA | 24 |
| PT002 | 9.122 | 1.557 | NB | 38 |
| PT003 | 9.039 | 1.711 | NB | 41 |
| PT004 | 6.366 | 1.525 | NB | 38 |
| PT006 | 8.118 | 1.698 | NB | 32 |
| PM1 | 6.937 | 21.294 | n.a., PHM | n.a. |
| PM2 | 7.689 | 38.615 | SA, PHM | 9 |
| PM3 | 8.496 | 112.240 | SA, HA | 7 |
| PM4 | 6.020 | 3.872 | SA, HA | 9 |
| BM1 | 7.732 | 24.395 | n.a., CHM | n.a. |
| BM2 | 5.142 | 34.784 | n.a., CHM | 9 |
| BM3 | 6.161 | 62.570 | n.a., CHM | n.a. |
| BM4 | 6.445 | 35.189 | SA, CHM | 6 |

RQ: relative quantification; CGA: calendar gestational age; SA: spontaneous abortion, AR: abruptio; NB: normal birth; PHM: partial hydatidiform mole; HA: hydropic abortion; CHM: complete hydatidiform mole; n.a.: not available.